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(54) Title: <b>DNA ENCODING THE VERTEBRATE HOMOLOG OF HEDGEHOG, VHH-1, EXPRESSED BY THE NOTOCHORD, AND USES THEREOF</b>			
(57) Abstract <p>This invention provides an isolated nucleic acid molecule encoding a vhh-1 protein, an isolated protein which is a vhh-1 protein, vectors comprising an isolated nucleic acid molecule encoding a vhh-1 protein, mammalian cells comprising such vectors, antibodies directed to a vhh-1 protein, nucleic acid probes useful for detecting a nucleic acid molecule encoding a vhh-1 protein, pharmaceutical compositions related to the vhh-1 proteins, nonhuman transgenic animals which express a normal or a mutant vhh-1 protein. This invention further provides methods for inducing differentiation of floor plate cell, motor neuron, generating ventral neurons and treatments for alleviating abnormalities associated with the vhh-1 protein.</p>			

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DNA ENCODING THE VERTEBRATE HOMOLOG OF HEDGEHOG, Vhh-1,  
EXPRESSED BY THE NOTOCHORD, AND USES THEREOF

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This application is a continuation-in-part of United States Patent Application Serial No. 08/202,040, filed February 25, 1994, the contents of which are hereby incorporated by reference.

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The invention disclosed herein was made with U.S. Government support under Grant Number NS-30532 from the National Institute of Health, U.S. Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

15

Background of the Invention

Throughout this application various publications are referred to by partial citations within parenthesis. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications, in their entireties, are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

20

In vertebrate embryos, the neural tube displays distinct cell types at defined dorsoventral positions. Floor plate cells differentiate at the ventral midline; motor neurons appear in ventrolateral regions; and sensory relay neurons, neural crest, and roof plate cells appear dorsally. The generation of cell pattern in the neural tube depends on signals that derive from surrounding tissues. A clear example of this is the influence of axial mesoderm on the development of ventral cell types.

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The differentiation of floor plate cells, motor neurons, and other ventral cell types requires inductive signals from axial mesodermal cells of the notochord. In the absence of the notochord, floor plate cells and motor neurons do not differentiate (Placzek et al., 1990b; Bovolenta and Dodd, 1991; Clarke et al., 1991; van Straaten and Hekking, 1991; Yamada et al., 1991; Ruiz i Altaba, 1992; Goulding et al., 1993; Ruiz i Altaba et al., 1993a; Halpern et al., 1993). Conversely, notochord grafts can induce the ectopic differentiation of floor plate cells and motor neurons in vivo and in vitro (van Straaten et al., 1988; Placzek et al., 1990b, 1991, 1993, Yamada et al., 1991, 1993; Ruiz i Altaba, 1992; Goulding et al., 1993). Floor plate cells themselves also possess both floor plate and motor neuron inducing activity (Yamada et al., 1991, 1993; Hatta et al., 1991; Placzek et al., 1993). In vitro assays have provided evidence that floor plate induction requires a contact-mediated signal, whereas motor neurons can be induced by diffusible signals (Yamada et al., 1993; Placzek et al., 1990b, 1993).

The differentiation of floor plate cells and motor neurons is associated with the expression of different classes of transcription factors. Floor plate cells express three members of the hepatocyte nuclear factor *HNF-3/fork head* gene family (Weigel and Jackie, 1990; Lai et al., 1991): *Pintallavis* (*XFKH1/XFD1/1*), *HNF-3 $\beta$* , and *HNF-3 $\alpha$*  (Dirksen and Jamrich, 1992; Knochel et al., 1992; Ruiz i Altaba and Jessell, 1992; Bolce et al., 1993; Monaghan et al., 1993; Ruiz i Altaba et al., 1993a; Sasaki and Hogan, 1993; Strahle et al., 1993). Ectopic expression of *Pintallavis* and *HNF-3 $\beta$*  leads to the appearance of floor plate markers in cells in the dorsal region of the neural tube (Ruiz i Altaba et al., 1992, 1993b; A.R.A. et al., unpublished data; Sasaki and Hogan,



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1994), suggesting that members of this family may specify floor plate cell fate. The differentiation of motor neurons is associated with expression of *islet-1*, a member of the LIM homeobox gene family (Ericson et al., 1992; Yamada et al., 1993). In addition to their possible functions in cell fate determination, these transcription factors provide markers that can be used in conjunction with cell surface molecules to monitor floor plate and motor neuron differentiation.

Cell patterning in the dorsal neural tube appears to be regulated by members of two families of secreted proteins that also have prominent roles in insect development. The transforming growth factor  $\beta$  (TGF $\beta$ ) family member *dorsalin-1* is expressed in the dorsal neural tube and can induce the differentiation of neural crest cells in neural plate explants in vitro (Basler et al., 1993). Members of the *wnt* family are also expressed in the dorsal neural tube (Roelink and Nusse, 1991; Nusse and Varmus, 1992; Parr et al., 1993). In *Drosophila*, the TGF $\beta$  family member *decapentaplegic* (*dpp*) regulates the dorsoventral pattern of the *Drosophila* embryo (see Ferguson and Anderson, 1992) and the differentiation and patterning of cells in imaginal discs (Spencer et al., 1982; Posakony et al., 1991; Campbell et al., 1993; Heberlein et al., 1993). Similarly, *wingless* (*wg*), a member of the *wnt* gene family, controls cell fates during segmentation and imaginal disc development (Morata and Lawrence, 1977; Nusslein-Volhard and Wieschaus, 1980; Baker, 1988; Martinez-Arias et al., 1988; Struhl and Basler, 1993).

A third *Drosophila* gene important in the specification of cell identity is *hedgehog* (*hh*) (Nusslein-Volhard and Wieschaus, 1980). *hh* acts with *dpp* and *wg* to control

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cell fate and pattern during segmentation and imaginal disc development (Hidalgo and Ingham, 1990; Ingham, 1993; Ma et al., 1993; Heberlein et al., 1993; Basler and Struhl, 1994; Heemskerk and DiNardo, 1994). *hh* encodes  
5 a novel protein (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993) that enters the secretory pathway (Lee et al., 1992), and genetic evidence indicates the *hh* function is not cell autonomous (Mohler, 1988; Heberlein et al., 1993; Ma et al., 1993; Basler and Struhl, 1994), consistent with the possibility  
10 that *hh* acts as a signaling molecule.

The importance of *hh* in cell patterning in insects prompted applicants to search for vertebrate homologs and  
15 to examine their potential functions during early neural development. Applicants disclose here the cloning of a vertebrate homolog of *hh*, *vhh-1*, from rat. Recent independent studies have identified a vertebrate homolog of *hh*, *sonic hedgehog (shh)*, that is closely related to  
20 *vhh-1* and appears to regulate cell patterning in the neural tube and limb bud (Echelard et al., 1993; Krauss et al., 1993, Riddle et al., 1993). Here, applicants present evidence that *vhh-1* is involved in the induction of ventral neural cell types. *vhh-1* is expressed in  
25 midline structures (in particular, the node, notochord, and floor plate) at a time when these cells have inducing activity. COS cells expressing the rat *vhh-1* gene induce floor plate and motor neuron differentiation in neural plate explants in vitro. Moreover, widespread  
30 expression of the rat *vhh-1* gene in frog embryos leads to ectopic expression of the floor plate markers in the neural tube. These results suggest that *vhh-1* expression in the notochord provides an inductive signal that is involved in the differentiation of floor plate cells,  
35 motor neurons, and possibly other cell types in the

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ventral neural tube.

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Summary of the Invention

This invention provides an isolated nucleic acid molecule encoding a vertebrate vhh-1 protein. In one embodiment of this invention, the nucleic acid molecule encoding a frog vhh-1 protein. In another embodiment, the nucleic acid molecule encoding a mammalian vhh-1 protein. In a further embodiment, the nucleic acid molecule encoding a rat vhh-1 protein. In a still further embodiment, the nucleic acid molecule encoding a human vhh-1 protein.

This invention provides a nucleic acid molecule comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a vertebrate vhh-1 protein.

This invention also provides monoclonal and polyclonal antibodies directed to a vhh-1 protein.

This invention provides a transgenic, nonhuman mammal comprising the isolated nucleic acid molecule encoding a vhh-1 protein.

This invention provides a method of producing a purified vertebrate vhh-1 protein which comprises: (a) inserting nucleic acid molecule encoding the vertebrate vhh-1 protein in a suitable vector; (b) introducing the resulting vector in a suitable host cell; (c) selecting the introduced host cell for the expression of the vertebrate vhh-1 protein; (d) culturing the selected cell to produce the vhh-1 protein; and (e) recovering the vhh-1 protein produced.

This invention provides a method of inducing the

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differentiation of floor plate cells comprising contacting floor plate cells with a purified vertebrate vhh-1 protein at a concentration effective to induce the differentiation of floor plate cells.

5

This invention provides a method of inducing the differentiation of floor plate cells in a subject comprising administering to the subject a purified vertebrate vhh-1 protein at an amount effective to induce the differentiation of floor plate cells in the subject.

10

This invention provides a method of inducing the differentiation of motor neuron comprising contacting the floor plate cells with a purified vertebrate vhh-1 protein at a concentration effective to induce the differentiation of motor neuron.

15

This invention provides a method of inducing the differentiation of motor neuron in a subject comprising administering to the subject a purified vertebrate vhh-1 protein at an amount effective to induce the differentiation of motor neuron in the subject.

20

This invention provides a method of generating ventral neurons comprising contacting progenitor cells with a purified vertebrate vhh-1 protein at a concentration effective to generate ventral neurons.

25

This invention provides a method of generating ventral neurons from progenitor cells in a subject comprising administering to the subject a purified vertebrate vhh-1 protein at an amount effective to generate ventral neurons from progenitor cells in the subject.

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This invention provides a pharmaceutical composition

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comprising a vertebrate vhh-1 protein and a pharmaceutically acceptable carrier. In an embodiment, the vhh-protein is a rat protein. In another embodiment, the vhh-protein is a human protein.

5

This invention provides a method for generating motor neurons from undifferentiated precursor neurons consisting of introducing an amount of a pharmaceutical composition comprising the human vhh-1 protein effective to generate motor neurons from undifferentiated precursor neurons. The generation of motor neurons can alleviate acute nervous system injury or chronic neurodegenerative diseases, such as Amyotrophic lateral sclerosis (ALS).

10

15

This invention provides a method of generating motor neurons from undifferentiated precursor neurons wherein the acute nervous system injury is localized to specific central axons which comprises surgical implantation of a pharmaceutical compound comprising the human vhh-1 protein and a pharmaceutically acceptable carrier effective to generate motor neurons from undifferentiated motor neurons located proximal to the injured axon(s).

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**Brief Description of the Figures****Figure 1-1, 1-2 and 1-3**

DNA Sequence of Rat Vhh-1 Protein with Corresponding  
Deduced Amino Acid Sequence.

**Figure 2A and 2A-2**

Deduced Amino Acid Sequences of Zebrafish and Rat  
Homologs of the Drosophila Hh Protein alignment of the  
zebrafish (Z1 vhh) and rat (R vhh) proteins with the  
Drosophila hh protein. Residues identical in all  
sequences are shown in bold. Gaps introduced to optimize  
the alignment are shown by ellipses. The vhh-1 sequence  
shows no homology with other proteins in the National  
Center for Biotechnology Information blast peptide  
sequence data base with the exception of residues 113-211,  
which show 39% conservation with the outer surface  
protein A of Borella burgdorferi, a lyme disease  
spirochete (Eiffert et al., 1992).

**Figure 2B**

Analysis of the hydrophilicity (Kyle and Doolittle, 1982)  
of the zebrafish and rat proteins. The NH<sub>2</sub>-terminus of  
the protein is to the left. Negative values indicate  
hydrophobic residues. The NH<sub>2</sub>-terminal hydrophobic region  
is likely to serve as a signal sequence (von Heijne,  
1985). Immediately following the putative signal  
sequence cleavage site is a basic region that conforms to  
the requirements for a heparin-binding site (Cardin and  
Weintraub, 1989).

**Figure 3A**

Localization of Rat vhh-1 mRNA by In Situ Hybridization  
vhh-1 mRNA expression in an E9.5 rat embryo. Labeled  
cells are found in the node (nd) and in the axial

-10-

mesoderm laid down at the midline of the embryo in the wake of the node. Anterior is up.

Scale bar is 165  $\mu$ m.

5 **Figure 3B**

Localization of *vhh-1* mRNA expression in an E10.5 rat embryo shown in side view *vhh-1* mRNA expression is present in the notochord (n in [C-E]) and in floor plate cells in more rostral regions of the spinal cord, hindbrain (h), and midbrain (m). Cells in the ventral diencephalon (d) also express *vhh-1* mRNA at high levels. In addition, a group of cells in the dorsal midbrain express *vhh-1* mRNA. Endodermal cells in the gut (g) also express the gene. At later stages a small group of cells in the rostral telencephalon also express *vhh-1* mRNA (data not shown).

Scale bar is 400 $\mu$ m.

**Figure 3C**

20 Cross section showing the neural plate and surrounding tissues in an E10 rat embryo. *vhh-1* mRNA expression is confined to a group of cells that lie under the midline of the neural plate.

Scale bar is 100 $\mu$ m.

25

**Figure 3D**

Cross section showing the neural plate and surrounding tissues in an E10 rat embryo. *vhh-1* mRNA expression is confined to the notochord (n).

30 Scale bar is 100 $\mu$ m.

**Figure 3E**

Cross section through an E11 rat embryo showing the spinal cord and surrounding tissues. *vhh-1* mRNA expression is detected in cells at the ventral midline of

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the spinal cord, corresponding to the floor plate (f) and to the notochord (n), which by this stage is displaced from the ventral midline of the nervous system. The border of the spinal cord is marked.

5 Scale bar is 180  $\mu$ m.

#### Figure 4A

Ectopic Expression of F-Spondin and *HNF-3 $\beta$*  in the Dorsal Neural Tube of Frog Embryos injected with a Plasmid  
10 Expressing Rat *vhh-1*. Cross section of neurula stage (approximately stage 16) *Xenopus* embryo expressing rat *vhh-1* mRNA from a plasmid driven by a CMV promoter. The rat *vhh-1* gene is detected predominantly in one half of the neural plate. Lateral arrows denote the lateral  
15 extent of the neural plate. Abbreviations: np. neural plate: n, notochord, s, somite.

#### Figure 4B

Lateral views of tadpole stage (approximately stage 34) embryos showing the pattern of F-spondin mRNA expression  
20 in an embryo injected with CMV plasmid encoding antisense *vhh-1*. F-spondin is expressed in the floor plate (fp) at the ventral midline of the neural tube and in the hypochord (h) located ventral to the notochord (n).  
25 Scale bar is 200  $\mu$ m.

#### Figure 4C

Lateral views of tadpole stage (approximately stage 34) embryos showing the pattern of F-spondin mRNA expression  
30 in an embryo injected with CMV plasmid encoding sense *vhh-1*. Ectopic expression of F-spondin mRNA is detected in the dorsal neural tube and in the dorsal ventricular zone adjacent to the floor plate (first and last arrowheads) (Ruiz i Altaba et al. 1993a). Ectopic F-  
35 spondin expression occurs in the posterior hindbrain and

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in the spinal cord.

Scale bar is 200  $\mu$ m.

**Figure 4D**

5 Cross section of tadpole stage (approximately stages 32-  
36) embryos injected with CMV plasmid encoding antisense  
vhh-1 and showing the expression of F-spondin mRNA.  
Embryos injected with CMV plasmids encoding antisense  
10 vhh-1 show a normal pattern of F-spondin mRNA expression,  
restricted to the floor plate (fp).  
Scale bar is 10  $\mu$ m.

**Figure 4E**

15 Cross section of tadpole stage (approximately stages 32-  
36) embryos injected with CMV plasmid encoding sense vhh-  
1 and showing the expression of F-spondin mRNA. Ectopic  
expression of F-spondin in embryos injected with CMV  
plasmids encoding sense vhh-1 is detected in roof plate  
cells in the hindbrain.  
20 Scale bar is 10  $\mu$ m.

**Figure 4F**

25 Cross section of tadpole stage (approximately stages 32-  
36) embryos injected with CMV plasmid encoding sense vhh-  
1 and showing the expression of F-spondin mRNA. Ectopic  
expression of F-spondin in embryos injected with CMV  
plasmids encoding sense vhh-1 is detected in the roof  
plate cells of the spinal cord.  
Scale bar is 10  $\mu$ m.

30

**Figure 4G**

35 Cross section of tadpole stage (approximately stages 32-  
36) embryos injected with CMV plasmid encoding antisense  
vhh-1 and showing the expression of HNF-3 $\beta$  protein.  
Embryos injected with a CMV plasmid encoding antisense

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vhh-1 show the normal pattern of HNF-3 $\beta$  protein expression, restricted to the floor plate (fp).  
Scale bar is 10  $\mu$ m.

5     **Figure 4H**

Cross section of tadpole stage (approximately stages 32-36) embryos injected with CMV plasmid encoding sense vhh-1 and showing the expression of HNF-3 $\beta$  protein. Ectopic expression of HNF-3 $\beta$  protein in the roof plate of the  
10   hindbrain (H) is detected in embryos expressing vhh-1 mRNA.  
Scale bar is 10  $\mu$ m.

**Figure 4I**

15   Cross section of tadpole stage (approximately stages 32-36) embryos injected with CMV plasmid encoding sense vhh-1 and showing the expression of HNF-3 $\beta$  protein. Ectopic expression of HNF-3 $\beta$  protein in the roof plate of the spinal cord is detected in embryos expressing vhh-1 mRNA.  
20   HNF-3 $\beta$  protein expression is also detected in very low levels in the notochord (n). Ectopic expression of these floor plate markers was also detected in the dorsal midbrain (data not shown).  
Scale bar is 10  $\mu$ m.

25

**Figure 5A**

Induction of Floor Plate differentiation in neural plant explants by vhh-1. Pattern of expression of the FP3 antigen in a cross section of the ventral region of an  
30   E11 rat spiral cord. FP3 expression is restricted to floor plate cells (f). The notochord (h) is unlabeled.  
Scale bar is 35 $\mu$ m.

**Figure 5B**

35   Induction of Floor Plate differentiation in neural plant

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explants by vhh-1. Pattern of expression of the FP4 antigen in a cross section of the ventral region of an E11 rat spinal cord. FP4 expression in the spinal cord is restricted to floor plate cells (f). The notochord (n) also expresses FP4.  
Scale bar is 35 $\mu$ m.

**Figure 5C**

Induction of Floor Plate differentiation in neural plant explants by vhh-1. Expression of FP3 by cells in rat neural plate explants that have been grown in contact with stage b chick notochord for 96 hours. Neural cells in proximity to the notochord express FP3.  
Scale bar is 45  $\mu$ m.

**Figure 5D**

Induction of Floor Plate differentiation in neural plant explants by vhh-1. Expression of FP4 by cells in rat neural plate explants grown in contact with stage 6 chick notochord for 96 hours. Neural cells in proximity to the notochord express FP4.  
Scale bar is 45  $\mu$ m.

**Figure 5E**

Induction of Floor Plate differentiation in neural plant explants by vhh-1. Phase-contrast micrograph showing expression of FP3 in neural plate cells grown in contact with COS cells transfected with cDNA encoding sense vhh-1. Intense expression of FP3 is detected at regions of contact between the neural plate explant and COS cell aggregate.  
Scale bar is 50  $\mu$ m.

**Figure 5F**

Induction of Floor Plate differentiation in neural plant

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explants by vhh-1. Fluorescence micrograph showing expression of FP3 in neural plate cells grown in contact with COS cells transfected with cDNA encoding sense vhh-1. Intense expression of FP3 is detected at regions of contact between the neural plate explant and COS cell aggregate.

Scale bar is 50  $\mu$ m.

**Figure 5G**

Induction of Floor Plate differentiation in neural plant explants by vhh-1. Phase-contrast micrograph showing expression of FP4 in neural plate cells grown in contact with COS cells transfected with cDNA encoding sense vhh-1. FP4 expression is detected at regions of contact between the neural plate (np) explant and COS cells (c). The junction between COS cells and neural plate explant is shown by the dotted line.

Scale bar is 60  $\mu$ m.

**Figure 5H**

Induction of Floor Plate differentiation in neural plant explants by vhh-1. Fluorescence micrograph showing expression of FP4 in neural plate cells grown in contact with COS cells transfected with cDNA encoding sense vhh-1. FP4 expression is detected at regions of contact between the neural plate (np) explant and COS cells (c). The junction between COS cells and neural plate explant is shown by the dotted line.

Scale bar is 60  $\mu$ m.

**Figure 5J**

Induction of Floor Plate differentiation in neural plant explants by vhh-1. Neural plate explants grown in contact with COS cells transfected with cDNA encoding antisense vhh-1 and labeled with anti-FP3 antibodies.

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The FP3 antigen is not expressed.

Scale bar is 60 $\mu$ m.

**Figure 5K**

- 5 Induction of Floor Plate differentiation in neural plate explants by vhh-1. Neural plate explants grown in contact with COS cells transfected with cDNA encoding antisense vhh-1 and labeled with anti-FP4 antibodies. The FP4 antigen is not expressed.
- 10 Scale bar is 60 $\mu$ m.

**Figure 6A**

- Induction of Motor Neuron Differentiation in Neural Explants by vhh-1. Section through a stage 17 chick spinal cord showing the expression of Islet-1<sup>+</sup> motor neurons in ventral spinal cord. Islet-1<sup>+</sup> cells are also detected in dorsal root ganglion neurons located next to the spinal cord.
- 15
- Scale bar is 70 $\mu$ m.

20

**Figure 6B**

- Induction of Motor Neuron Differentiation in Neural Explants by vhh-1. Phase-contrast micrographs explants grown for 44 hours on a monolayer of COS cells transfected with cDNA encoding sense vhh-1. The field shows three explants containing Islet-1<sup>+</sup> cells. COS cells nuclei (COS) visible under the neural plate explants. The border between the neural plate explants and COS cell monolayer is shown.
- 25
- 30 Scale bar is 70 $\mu$ m.

**Figure 6C**

- Induction of Motor Neuron Differentiation in Neural Explants by vhh-1. Florescence micrographs explants grown for 44 hours on a monolayer of COS cells
- 35

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transfected with cDNA encoding sense vhh-1. The field shows three explants containing Islet-1<sup>+</sup> cells. COS cells nuclei (COS) visible under the neural plate explants. The border between the neural plate explants and COS cell monolayer is shown. :  
5 Scale bar is 70 $\mu$ m.

**Figure 6D**

Induction of Motor Neuron Differentiation in Neural  
10 Explants by vhh-1. Section through a stage 17 chick spinal cord showing the distribution of SC1 in floor plate cells (f), motor neurons (m), and notochord (n). Scale bar is 70 $\mu$ m.

**Figure 6E**

Induction of Motor Neuron Differentiation in Neural  
Explants by vhh-1. Confocal image of a single field in  
a chick neural plate explant grown 44 hours on COS cells  
transfected with the vhh-1 gene and labelled with  
20 antibodies against SC1. All SC1<sup>+</sup> cells express Islet-1  
in their nuclei (Compare with Figure 5F). Clusters of  
SC1<sup>+</sup>/Islet-1<sup>+</sup> cells were not detected in these explants  
(data not shown).  
Scale bar is 13 $\mu$ m.

25

**Figure 6F**

Induction of Motor Neuron Differentiation in Neural  
Explants by vhh-1. Confocal image of a single field in  
a chick neural plate explant grown 44 hours on COS cells  
transfected with the vhh-1 gene and labelled with  
30 antibodies against Islet-1.  
Scale bar is 13 $\mu$ m.

**Figure 6G**

35 Neural plate explants grown for 48 hours on a monolayer

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of COS cells transfected with a gene encoding antisense vhh-1 and labelled with anti-Islet-1 antibodies. No expression of Islet-1 is detected.

Scale bar is 70 $\mu$ m.

5

**Figure 6H**

Neural plate explants grown for 48 hours on a monolayer of COS cells transfected with a gene encoding antisense vhh-1 and labelled with anti-SC1 antibodies. No expression of SC1 is detected. This image is of a confocal section through an explant.

10

Scale bar is 13 $\mu$ m.

**Figure 7A**

Cells in Posterior Limb Bud Mesenchyme Express mRNA Encoding vhh-1 and Can Enduce Floor Plate Differentiation in Neural Plate Explants. Section through limb bud of an E11 rat embryo showing expression of mRNA encoding vhh-1 in mesenchymal cells located in the posterior (p) region of the limb bud. Mesenchymal cells in the anterior (a) region of the cell do not express mRNA encoding vhh-1. Ectodermal cells do not express vhh-1 mRNA.

20

Scale bar is 270 $\mu$ m.

25

**Figure 7B**

Cells in Posterior Limb Bud Mesenchyme Express mRNA Encoding vhh-1 and Can Enduce Floor Plate Differentiation in Neural Plate Explants. Phase-contrast micrograph showing expression of FP3 by neural plate cells grown in contact with chick posterior limb mesenchyme. Neural plate cells express FP3.

30

Scale bar is 60 $\mu$ m.

**Figure 7C**

Cells in Posterior Limb Bud Mesenchyme Express mRNA

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Encoding *vhh-1* and Can Educe Floor Plate Differentiation  
in Neural Plate Explants. Fluorescence micrograph  
showing expression of FP3 by neural plate cells grown in  
contact with chick posterior limb mesenchyme. Neural  
plate cells express FP3.  
Scale bar is 60 $\mu$ m.

**Figure 7D**

Induction of Motor Neuron Differentiation in Neural  
Explants by *vhh-1*. Phase-contrast micrograph of neural  
plate explants grown in contact with anterior limb bud  
mesenchyme. No expression of FP3 is detected.  
Scale bar is 60 $\mu$ m.

**Figure 7E**

Induction of Motor Neuron Differentiation in Neural  
Explants by *vhh-1*. Fluorescence micrograph of neural  
plate explants grown in contact with anterior limb bud  
mesenchyme. No expression of FP3 is detected.  
Scale bar is 60 $\mu$ m.

**Figure 8A**

*vhh-1/shh* and *Islet-1* are expressed in Adjacent Ventral  
Domains in the Embryonic Chick Central Nervous System.  
(A) Sagittal view showing the domain of *vhh-1/shh*  
expression in the central nervous system of a HH stage  
18/19 chick embryo (shaded area). The dashed lines  
indicate the axial levels and planes of the sections  
shown in panels B-K.

(B-K) The domains of *vhh-1/shh* mRNA (blue-black) and  
*Islet-1* (brown) express in adjacent domains of the  
ventral CNS.

**Figure 8B**

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(B) A transverse section through the caudal rhombencephalon showing *vhh-1/shh* expression at the ventral midline in the floor plate and *Islet-1* expression, laterally, in motor neurons.

5

**Figure 8C**

(C) A sagittal section of the neural tube showing *vhh-1/shh* and *Islet-1* expression in the ventral mesencephalon, diencephalon and telencephalon. In the mesencephalon and rostral diencephalon, cells that express *Islet-1* are located adjacent to the ventral domain of expression of *vhh-1/shh*. *vhh-1/shh* expression is detected in the basal telencephalon, rostral to the optic chiasm (arrow head) and here, *Islet-1* cells are found ventral and rostral to the domain of *vhh-1/shh* expression. Note that there is a region at the rostral-most tip of the ventral diencephalon, abutting the optic chiasm, that does not express *vhh-1/shh*.

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**Figure 8D**

(D) A transverse section through the mid-diencephalon at the level of infundibulum (i). Cells that express *vhh-1/shh* form two bilateral stripes. Cells that express *Islet-1* are located at the lateral edge of the domain of *vhh-1/shh* expression. *Islet-1* cells are absent from the ventral midline at the level of the infundibulum. Cells at the ventral region of Rathke's pouch (r) express *Islet-1*.

30

**Figure 8E**

(E) In the rostral diencephalon at HH stage 13, cells that express *Islet-1* are interspersed with cells that express *vhh-1/shh*. The double labeling method does not resolve whether any cells coexpress *vhh-1/shh* and *Islet-1* at this stage.

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**Figure 8F**

(F) A transverse section through the mesencephalon showing ventral midline expression of *vhh-1* and *Islet-1*. At this axial level, a small number of *Islet-1* sensory neurons can also be detected dorsally, in the trigeminal mesencephalic nucleus.

**Figure 8G**

(G) Higher magnification of (F) showing that the domain of *vhh-1/shh* expression expands lateral to the midline and that *Islet-1* cells are located lateral to the midline domain of *vhh-1/shh* expression.

**Figure 8H**

(H) A transverse section at the level of the rostral diencephalon showing ventral midline expression of *vhh-1* and *Islet-1*.

**Figure 8I**

(I) Higher magnification of (H) showing the ventral midline of the rostral diencephalon. Both *vhh-1/shh* and *Islet-1* are expressed at the midline of the rostral diencephalon. *vhh-1/shh* is expressed in the ventricular zone whereas *Islet-1* cells are located basally.

**Figure 8J**

(J) A transverse section at the level of the caudal telencephalon showing *vhh-1/shh* and *Islet-1* cells in the floor of the telencephalon.

**Figure 8K**

(K) Higher magnification of (J). In the ventral telencephalon cells that express *vhh-1/shh* and *Islet-1* are more dispersed than at caudal regions of the ventral CNS. The lack of *vhh-1/shh* expression by cells at the

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ventral midline suture of the telencephalon is a consistent observation. Whole-mount in situ hybridization was performed using a chick *Islet-2* probe (Tsuchida et al., 1994). Chick *Islet-2* mRNA was not expressed at rhombencephalic, mesencephalic, diencephalic or telencephalic levels, indicating that immunoreactivity detected with the *Islet-1* antisera corresponds to the *Islet-1* protein (data not shown). Abbreviations: i: infundibulum, di: diencephalon, me: mesencephalon, te: telencephalon. Scale bar: B, G, I, K = 50  $\mu$ m; C, F, H, J = 200  $\mu$ m; D = 100  $\mu$ m, E = 25  $\mu$ m.

**Figure 9A**

(A) Diagram of a sagittal section of the neural tube of a HH stage 18/19 chick embryo showing the domains of expression of cell type markers, (i) summary diagram of the domains of expression *vhh-1/shh* (stippled) and *Islet-1* (red) derived from the whole-mount labeling shown in Figure 8. (ii) Summary diagram showing the coexpression of markers in *Islet-1*<sup>+</sup> neurons. In the rhombencephalon (r) and mesencephalon (m), ventral *Islet-1*<sup>+</sup> neurons coexpress the surface immunoglobulin protein SC1 (green domain). In the ventral diencephalon, *Islet-1*<sup>+</sup> neurons are absent from the most caudal region, although *Lim-1*<sup>+</sup> cells (brown) are expressed. In the region of the mid-diencephalon, rostral to the zona limitans interthalamica (Puelles et al., 1987), and also at the ventral midline of the rostral diencephalon, most *Islet-1*<sup>+</sup> neurons coexpress *Lim-1* (blue domain). In the intervening region of the mid-diencephalon above the infundibulum (i), *Islet-1* and *Lim-1* are expressed in separate but intermingled neuronal populations (domain indicated by brown and red stripes). In the ventral telencephalon, *Islet-1*<sup>+</sup> neurons (red domain) do not express SC1 or *Lim-1*. For simplicity, the domain of neuroepithelia *Lim-1*

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**Figure 9G, 9H, 9I**

(G, H, I), Ventral detail of a transverse section through the rostral diencephalon showing that Islet-1<sup>+</sup> neurons (I) (red) express Lim-1 (H) (green). (I) shows a double exposure of (G) and (H) to indicate the extent of overlap of labeled cells.

**Figure 9J**

(J) Ventral detail of a coronal section through the ventral telencephalon showing that Islet-1<sup>+</sup> neurons do not express Lim-1, as shown by the absence of yellow cells in this double exposure of Islet-1 (rhodamine) and Lim-1 (FITC). Abbreviations: r: rhombencephalon, m: mesencephalon, d: diencephalon, t: telencephalon and i: infundibulum. The sections shown in (B-J) are from HH stage 18-19 embryos. Scale bar: B = 160  $\mu$ m; C, E-I = 25  $\mu$ m; and D, J = 20  $\mu$ m.

**Figure 10A**

*vhh-1/shh* induces Islet-1<sup>+</sup> Neurons in Explants Derived from Different Rostrocaudal Levels of the Neural Plate. (A) Expression of *vhh-1/shh* mRNA in the cells at the midline of a HH stage 6 chick embryo shown by whole mount in situ hybridization. Sections through such embryos shows that *vhh-1/shh* mRNA is expressed both in neural ectoderm and in the underlying mesoderm (data not shown). The position of the prospective telencephalic (T), diencephalic (D) and rhombencephalic (R) regions of the neural plate isolated for in vitro assays is indicated. The head-fold is at the top and the approximate neuroectodermal/ectodermal border is indicated by a dashed line. Dotted line indicates approximate border of the epiblast. Immunofluorescence micrographs in B-M show explants cultivated for approximately 65 hours on COS cells transfected with antisense or sense *vhh-1* cDNA.

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expression that occupies the entire dorsoventral extent of the mid-diencephalon, rostral to the zona limitans interthalamica is not depicted in this diagram. (iii) Summary diagram showing the ventral domain of expression of Nkx 2.1 protein. Small arrows indicate the plane of sections shown in panels B-J.

**Figure 9B**

Ventral detail of a transverse section through the mesencephalon showing that motor neurons of oculomotor (III) nucleus coexpress Islet-1 (red) and SC1 (green). Oculomotor neurons are the most rostrally located group of Islet-1<sup>+</sup> cells that coexpress SC1. Somatic visceral and brachial motor neurons at more caudal levels also express SC1 (see also Simon et al., 1994).

**Figure 9C**

(C) Ventral detail of a transverse section through the rostral diencephalon showing that Islet-1<sup>+</sup> neurons do not express SC1. SC1-labeled axons in (C) derive from neurons located more rostrally that do not express Islet-1.

**Figure 9D**

(D) Detail of a transverse section through the ventral telencephalon showing expression of Nkx 2.1 in most cells.

**Figure 9E, 9F**

(E, F) Detail of a transverse section through the lateral region of the mid-diencephalon dorsal to the infundibulum (see Fig. 8D for a low power view) showing that all virtually all undifferentiated neuroepithelial cells express Lim-1 at low levels (F) and that Islet-1<sup>+</sup> neurons (E) (red) also coexpress Lim-1 (yellow cells in (F)).

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**Figure 11A and 11B**

SCI Expression Distinguishes the Islet-1<sup>+</sup> Neurons Induced by vhh-1/shh in Explants Derived from Rostral and Caudal Levels of the Neural Plate. (A, B) Immunofluorescence micrographs of a section through a rhombencephalic level neural plate explant exposed to vhh-1/shh. Double-label images of the same section shows that Islet-1<sup>+</sup> cells (A) express SCI (B). Arrows in (A) and (B) indicate the same cell.

**Figure 11C and 11D**

(C, D) Patches of cells in rhombencephalic level explants express SCI (D) but not Islet-1 (C). These SCI<sup>+</sup> cells coexpress FPl (data not shown) indicating that they are floor plate cells.

**Figure 11E and 11F**

(E, F), Immunofluorescence micrographs of a section through a diencephalic level neural plate explant exposed to vhh-1/shh. Islet-1<sup>+</sup> cells (E) do not coexpress SCI (F).

**Figure 11G and 11H**

(G, H) Immunofluorescence micrographs of a section through a telencephalic level neural plate explant exposed to vhh-1/shh. Islet-1<sup>+</sup> cells (G) do not express SCI (H). Scale bar: A, B, E-H = 10  $\mu$ m and C, D = 25  $\mu$ m.

**Figure 12A**

Expression of Nkx 2.1 and Lim-1 Distinguishes Ventral Neurons Induced by vhh-1/shh in Diencephalic and Telencephalic Level Neural Plate Explants.

(A-C) Expression of Nkx 2.1 in neural plate explants from different axial levels exposed to vhh-1/shh. (A) Absence

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**Figure 10B and 10C**

(B, C) Section of a rhombencephalic level explant grown on COS cells transfected with antisense *vhh-1/shh*. No Islet-1<sup>+</sup> cells are detected (B) even though  $\beta$ -tubulin<sup>+</sup> neurons have differentiated (C).

**Figure 10D and 10E**

(D, E) Section of a rhombencephalic level explant grown on COS cells transfected with sense *vhh-1/shh*. Numerous Islet-1<sup>+</sup> cells are detected (D) virtually all of which coexpress  $\beta$ -tubulin (E).

**Figure 10F and 10G**

(F, G) Section of a diencephalic level explant grown on COS cells transfected with antisense *vhh-1/shh*. No Islet-1<sup>+</sup> cells are detected (F) even though  $\beta$ -tubulin<sup>+</sup> neurons are present (G).

**Figure 10H and 10I**

(H, I) Section of a diencephalic level explant grown on COS cells transfected with sense *vhh-1/shh*. Numerous Islet-1<sup>+</sup> cells are present, and these coexpress  $\beta$ -tubulin (I).

**Figure 10J and 10K**

(J, K) Section through a telencephalic level explant grown on COS cells transfected with antisense *vhh-1/shh*. No Islet-1<sup>+</sup> cells are detected (J) despite the differentiation of  $\beta$ -tubulin<sup>+</sup> neurons (K).

**Figure 10L and 10M**

(L, M) Section of a telencephalic level explant grown on COS cells transfected with sense *vhh-1/shh*. Numerous Islet-1<sup>+</sup> cells are present (L), and these coexpress  $\beta$ -tubulin (M). Scale bar: A = 250  $\mu$ m and B-M = 25  $\mu$ m.



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explants. Scale bar: 20  $\mu$ m.

**Figure 13A**

Floor plate and Midline Rostral Diencephalic Cells Mimic  
5 the Ability of vhh-1/shh to Induce Ventral Neurons at  
Different Levels of the Neuraxis.

(A) Islet-1<sup>+</sup> neurons are induced by floor plate in  
rhombencephalic level neural plate explants. These cells  
10 coexpress SC1 (data not shown).

**Figure 13B**

(B) Nkx 2.1 is not induced by floor plate in  
rhombencephalic level explants.  
15

**Figure 13C**

(C) Rostral diencephalic tissue induces Islet-1<sup>+</sup> cells  
(green) in telencephalic level neural plate explants.  
Diencephalic tissue of murine origin is delineated by  
20 anti-nestin immunoreactivity (red) and contains a few  
Islet-1<sup>+</sup> neurons (yellow cells). The induced  
telencephalic Islet-1<sup>+</sup> neurons do not express SC1 (data  
not shown). About 10-20% of cells in the telencephalic  
explants expressed Islet-1.

25

**Figure 13D and 13E**

(D, E) Floor plate tissue induces Islet-1<sup>+</sup> neurons (D) in  
telencephalic level explants. These neurons do not  
coexpress SC1 (E). The floor plate tissue is not  
30 depicted in this field.

**Figure 13F**

(F) Floor plate induces Nkx 2.1<sup>+</sup> cells in telencephalic  
level explants. Scale bar: A, B = 15  $\mu$ m, C = 30  $\mu$ m, D,  
35 E = 10  $\mu$ m and F = 12  $\mu$ m.

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of expression of Nkx 2.1 in a rhombencephalic level neural plate explant exposed to vhh-1/shh.

**Figure 12B**

- 5 (B) Expression of Nkx 2.1 in diencephalic level neural plate explant exposed to vhh-1/shh.

**Figure 12C**

- 10 (C) Expression of Nkx 2.1 in a telencephalic level neural plate explant exposed to vhh-1/shh. No expression of Nkx 2.1 was observed in neural plate explants that had not been exposed to vhh-1/shh (not shown).

**Figure 12D**

- 15 (D) Lim-1<sup>+</sup> cells are present in diencephalic level neural plate explants that have not been exposed to vhh-1/shh.

**Figure 12E and 12F**

- 20 (E, F) Many Islet-1<sup>+</sup> cells (E) in diencephalic level explants exposed to vhh-1/shh express Lim-1 (F). Arrows indicate some of the cells that coexpress Islet-1 and Lim-1. Note that Islet-1<sup>+</sup>/Lim-1<sup>+</sup> and Islet-1<sup>-</sup>/Lim-1<sup>+</sup> cells are also present.

**Figure 12G**

- 25 (G) No Lim-1<sup>+</sup> cells are detected in telencephalic level neural plate explants that have not been exposed to vhh-1/shh.

**Figure 12H and 12I**

- 30 (H, I) Islet-1<sup>+</sup> cells (H) in telencephalic level neural plate explants exposed to vhh-1/shh do not express Lim-1 (I). Note that no Lim-1<sup>+</sup> cells are present in telencephalic level explants even after exposure to vhh-1/shh. Similar results were obtained in over 20
- 35

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(n) do not express either gene when cultured alone for 36 h. Contact with the notochord (n + [i]) induces *HNF3 $\beta$*  and *Netrin-1* expression (upper bands).

5 **Figure 14F**

(F) RT-PCR analysis of *Isl-1*, *Isl-2* and *ChAT* mRNA induction by contact with the notochord. Intermediate neural plate explants ([i]) and notochord (n) do not express *Isl-1*, *Isl-2* or *ChAT* (8) when cultured alone for 10 36 h. Contact with the notochord (n + [i]) induces the expression of all three genes (upper bands). Lower bands marked by arrow indicate internal standards introduced to control for the efficiency of the RT-PCR reactions. Results in E and F were obtained from RNA from the same 15 set of explants. Similar results were obtained in 6 experiments.

**Figure 14G**

(G) Neural plate explants separated from the notochord by 20 a Nucleopore filter and grown in vitro for 36 h contain *Isl*<sup>+</sup> (green) but not *HNF3 $\beta$* <sup>+</sup> (red) cells.

**Figure 14H**

(H) *Isl*<sup>+</sup> cells (green) present in neural plate explants 25 grown transfilter to the notochord express *SC1* (red) indicating that they are motor neurons. Patches of *SC1*<sup>+</sup>/*Isl*<sup>+</sup> cells were not detected, indicating the absence of floor plate differentiation. Similar results were obtained in 4 separate experiments using either 30 Nucleopore or dialysis membrane filters. Scale bar: A, C, H = 20  $\mu$ m; B = 100  $\mu$ m; D,G = 33  $\mu$ m.

**Figure 15A**

COS Cells that Express *Shh/vhh-1* Exhibit Contact- 35 Dependent Floor Plate and Diffusible Motor Neuron-

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**Figure 14A**

Induction of Floor Plate and Motor Neuron Differentiation by the Notochord is Distinguished by Dependence on Cell Contact.

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(A) Neural plate explant grown for 36 h in the absence of the notochord and labelled with antibodies that detect HNF3 $\beta$  and Isl-1 and/or Isl-2 (Isl<sup>+</sup> cells). No HNF3 $\beta$ <sup>+</sup> or Isl<sup>+</sup> cells are detected.

10

**Figure 14B**

(B) Neural plate explant grown for 36 h in contact with notochord (n). HNF3 $\beta$ <sup>+</sup> (red) and Isl<sup>+</sup> (green) cells are induced. HNF3 $\beta$ <sup>+</sup> cells are located closer to the notochord/neural plate junction (----) than are Isl<sup>+</sup> cells.

15

**Figure 14C**

(C) Isl<sup>+</sup> cells (green) induced in neural plate explants by contact with the notochord coexpress the surface immunoglobulin-like protein SC1 (red). Patches of SC1<sup>+</sup> cells that do not express Isl proteins (arrowhead) correspond to floor plate cells (34).

20

**Figure 14D**

(D) Contact with the notochord induces Isl-2<sup>+</sup> cells (green) in neural plate explants. HNF3 $\beta$ <sup>+</sup> cells (red) are also induced.

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**Figure 14E**

(E) RT-PCR analysis of HNF3 $\beta$  and Netrin-1 mRNA induction by contact with the notochord. Lower bands marked by arrow indicate competitive templates introduced to control for the efficiency of the RT-PCR reactions. Intermediate neural plate explants ([i]) and notochord

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contain HNF3 $\beta$ , Isl-1 or Isl-2 cells (Table 2 and data not shown).

**Figure 15G**

5 (G) RT-PCR analysis of floor plate induction by *vhh-1*-transfected COS cells. *HNF3 $\beta$*  and *Netrin-1* expression is induced in neural plate explants grown in contact with *vhh-1*-transfected COS cells (lanes 1) but not with antisense *vhh-1*-transfected COS cells (lanes 2). *HNF3 $\beta$*   
10 and *Netrin-1* expression is not induced in neural plate explants grown at a distance from *vhh-1*-transfected (lanes 3) or antisense *vhh-1*-transfected (lanes 4) COS cells. In the same experiment, notochord grown in contact with neural plate explants induces both *HNF3 $\beta$*  and  
15 *Netrin-1* expression (lanes 5).

**Figure 15H**

(H) RT-PCR analysis of motor neuron induction by *vhh-1*-transfected COS cells. *Isl-1* and *ChAT* expression is  
20 induced in neural plate explants grown in contact with *vhh-1*-transfected COS cells (lanes 2). *Isl-1* and *ChAT* expression are also induced in neural plate explants grown at a distance from *vhh-1*-transfected COS cells (lanes 3). *Isl-1* and *ChAT* expression is not induced in  
25 neural plate explants exposed to COS cells transfected with antisense *vhh-1* (lanes 2 and 4). Notochord grown in contact with neural plate explants induces both *Isl-1* and *ChAT* (lanes 5). Results shown in Panels A-H have been replicated in 6 different experiments. Scale bar : A,  
30 D = 16  $\mu$ m; C, F = 33  $\mu$ m.

**Figure 16A**

Induction of Floor Plate and Motor Neuron Differentiation by Transfection of *vhh-1* into Neural Plate Explants.

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## Inducing Activities.

5 (A) Neural plate explant grown in contact with *vhh-1*-transfected COS cells for 36 h contains HNF3 $\beta$ <sup>+</sup> (red) and Isl<sup>+</sup> (green) cells. The two cell groups are intermingled. Apparent yellow cells represent the superimposition of two distinct nuclei in the confocal section.

## Figure 15B

10 (B) Isl<sup>+</sup> neurons (green) in neural plate explants grown in contact with *vhh-1*-transfected COS cells express SC1 (red). Isl<sup>+</sup> neurons that do not coexpress SC1 probably represent newly-differentiated motor neurons (34).

## 15 Figure 15C

(C) Many Isl-1<sup>+</sup> neurons in intermediate neural plate explants grown in contact with *vhh-1*-transfected COS cells coexpress Isl-2 (orange cells).

## 20 Figure 15D

(D) Neural plate explant separated from *vhh-1*-transfected COS cells in a collagen gel and grown for 36 h contains Isl<sup>+</sup> (green) but not HNF3 $\beta$ <sup>+</sup> (red) cells.

## 25 Figure 15E

(E) Isl<sup>+</sup> neurons (green) induced at a distance from *vhh-1*-transfected COS cells coexpress SC1 (red) and are motor neurons.

## 30 Figure 15F

(F) Isl-1<sup>+</sup> neurons (green) induced at a distance from *vhh-1*-transfected COS cells coexpress Isl-2 (red), as shown by orange-labeled nuclei. Intermediate neural plate explants grown in contact with or at a distance from COS  
35 cells transfected with antisense *vhh-1* cDNA did not

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possible mechanisms by which *shh/vhh-1* derived from the notochord (dark shading) could induce floor plate (FP) and motor neuron (MN) differentiation independently.

5 (A) Floor plate and motor neuron differentiation could be mediated by different fragments of *shh/vhh-1* that are generated by autoproteolysis (28). The amino terminal (N) fragment of hedgehog remains largely associated with the cell surface whereas the carboxy terminal fragment (C) is  
10 freely diffusible (28). Thus, in this diagram N is depicted as mediating the contact-dependent induction of floor plate differentiation and C, the longer range, contact-independent induction of motor neurons.

15 **Figure 17B**

(B) Floor plate and motor neuron differentiation could be mediated by different concentrations of the same molecular species of *shh/vhh-1*. Since neural plate cells that are located immediately above the notochord  
20 differentiate into floor plate cells, the diagram indicates that a high concentration of *shh/vhh-1* ( $\rightarrow$ ) is required to elicit floor plate differentiation. Lower concentrations of *shh/vhh-1* ( $---\rightarrow$ ) initiate motor neuron differentiation independent of floor plate  
25 differentiation.

**Figure 18A, 18B, 18C**

Embryonic midline expression of *vhh-1*, *Pintallavis*, *gooseoid*, and HNF-3 $\beta$ . All panels show Nomarski images  
30 of whole-mount in situ hybridizations (A-E, J-M, O, Q) or histological section (F-I, N, P) labeled with an antisense *vhh-1* RNA probe (A, D, F-H, J-N, Q), an antisense *Pintallavis* RNA probe (B, E, I), an antisense *gooseoid* RNA probe (C) or antibodies directed against  
35 HNF-3 $\beta$  (O, P).

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(A) RT-PCR analysis of floor plate and motor neuron marker expression in neural plate explants analyzed 48 h after transfection with a CMV *vhh-1*-transfected explants (*vhh-1*) but not in mock-transfected (-) explants. *Isl-1* was also detected in *vhh-1*-transfected neural plate explants grown in the absence of NT3 but at lower levels (data not shown). Cells that expressed *HNF3 $\beta$*  and *Isl* immunoreactivity could also be detected (data not shown) although there was an extremely high background, possibly because of cell damage as a consequence of the transfection protocol.

**Figure 16B**

(B) Time course of *HNF3 $\beta$*  and *Isl-1* expression in neural plate explants transfected with a CMV *vhh-1* cDNA expression construct. (i) In this experiment neither *Isl-1* nor *HNF3 $\beta$*  are expressed 10 h or 20 h after transfection (lanes 1 and 2) but are detected at 30 h and 40 h (lanes 3 and 4). *Netrin-1* and *Isl-2* are also expressed after 30 h (data not shown). (ii) In this experiment *Isl-1* expression is not apparent at 10 h (lane 1) and can first be detected at 22 h (lane 2). In contrast, *HNF3 $\beta$*  expression is not detected at either 22h or 24h (lanes 2 and 3) although the gene is expressed at 40 h (lane 4). Results showing that *Isl-1* expression occurs before or coincident with *HNF3 $\beta$*  expression were obtained in 4 separate experiments. In a further 3 experiments, *Isl-1* expression was detected although *HNF3 $\beta$*  could not be detected. *Isl-1* was also detected in *vhh-1*-transfected neural plate explants grown in the absence of NT3 (data not shown; see below).

**Figure 17A**

Independent Induction of Floor Plate and Motor Neuron Differentiation by *Shh/vhh-1*. Diagrams depict two



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(A-C) Expression of *vhh-1* (A) *Pintallavis* (B) and *goosecoid* (C) in early (stage 10) gastrula embryos. Note the absence of *vhh-1* mRNA from the early dorsal blastopore lip (dbp) or organizer region (A) which expresses *Pintallavis* and *goosecoid* (B, C). Panels show 5 vegetal views with dorsal side up (A, C) or slightly to the right (B).

**Figure 18D and 18E**

10 (D, E) *vhh-1* is expressed in cells of the notochord (n) as it forms but is absent from the future tailbud region, near the blastopore (bp; D). *Pintallavis*, in contrast, is expressed throughout the notochord, including cells near the blastopore (E). Both *vhh-1* and *Pintallavis* are 15 also expressed in the prechordal plate (pp) a: anterior, p: posterior. Panels show dorsal views with anterior end to the left.

**Figure 18F, 18G, 18H and 18I**

20 Transverse sections of midline regions of gastrula and neurula stage embryos labelled in whole mount with an antisense *vhh-1* RNA probe (F-H) or an antisense *Pintallavis* RNA probe (I). Expression of *vhh-1* is detected in notochord (n) but not in neural plate (np) 25 cells during early gastrula stages (stage approximately 11, F). Within the notochord, expression of *vhh-1* is confined mainly to dorsal cells that underly the neural plate. At late gastrula stages (stage approximately 12.5 - 13, G), expression of *vhh-1* within the notochord is 30 detected at high levels in the most dorsal cells and expression is also detected in cells of the deep (d) but not superficial (s) cells of the neural plate (Schroeder, 1970). At early neurula stages (stage approximately 15), *vhh-1* is expressed in median deep (md) neural plate cells 35 forming a triangle over the notochord (n) but not in

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adjacent intermediate deep (id) or median superficial (ms) cells (H). Levels of expression in the notochord are very low. Following neural tube closure (stage approximately 20) expression of *vhh-1* is still restricted to md cells (not shown). In older embryos (from stage approximately 24) md and ms cells intermix at the ventral midline of the neural tube and *vhh-1* expression is detected in all ventral midline cells of the floor plate (stage approximately 36; N). *Pintallavis* mRNA is also detected in deep (d) but not superficial (s) cells of the neural plate (I) and in midline endodermal cells (en) underlying the notochord which will form the hypochord. Note the even distribution of *Pintallavis* expression throughout the notochord in comparison to that of *vhh-1* shown in (F, G). s: somites. In all panels, dorsal side is up.

**Figure 18J, 18K, 18L and 18M**

(J-M) Expression of *vhh-1* mRNA in neurula (stage 15, J), tailbud (stages approximately 20, approximately 26, K and L) and tappole (stage approximately 36, M) embryos labelled in whole mount. At the early neurula stage (stage approximately 15, J), *vhh-1* is expressed in the floor plate (fp), prechordal plate mesoderm (pp) and adjacent anterior endoderm at high levels whereas its expression in the notochord (n) is lower than at earlier stages. Within the notochord there appears to be a gradual loss of *vhh-1* mRNA from anterior to posterior regions. *vhh-1* is also expressed in cells of the ventral forebrain overlying the prechordal plate. At early tailbud stages (stage approximately 20, K), *vhh-1* is detected at high levels in the floor plate of the hindbrain and midbrain (m), in the entire ventral diencephalon (d) and prechordal plate mesoderm (pp) which underlies the forebrain. *vhh-1* mRNA is also detected in

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pharyngeal endoderm (pe) anterior to the prechordal plate. No expression is detected in the notochord (n) or telencephalon (t). Note the sharp boundary between cells expressing *vhh-1* in the ventral diencephalon and those not expressing *vhh-1* in the ventral telencephalon. At late tailbud stages (stage approximately 26, L), *vhh-1* is still expressed in the floor plate (fp) and midline cells of the ventral diencephalon (vd) but not in the telencephalon (t). *vhh-1* expression is undetectable in the notochord (n) but it remains in the prechordal plate and in areas of the anterior endoderm (en). As the brain develops, there is expression in posterior diencephalic cells in more lateral areas (unlabelled arrow in L). Expression in the lateral diencephalon comprises a broad bilateral stripe. *vhh-1* expression is also observed in an anterior position, ventral to the telencephalon (t) and dorsal to the cement gland, corresponding to the olfactory placode (op).

Expression of *vhh-1* mRNA is detected in tadpoles (stage approximately 36, M) at high levels in the floor plate (fp) throughout its length, a dorsal-posterior diencephalic region and in broad bilateral diencephalic (d) stripes. At later stages, (stage >40) expression is detected in a small group of cells in the ventral telencephalon (not shown). *vhh-1* is reexpressed at tadpole stages in the notochord (n). The tailbud (tb) does not express *vhh-1* but expression is detected in cells forming the hypochord (located ventral to the notochord), notochord and floor plate as soon as these leave the tailbud (not shown). In the head, *vhh-1* is widely expressed in the gill endoderm (ge) and in the frontonasal region, adjacent to the telencephalon (t). At later stages (stage approximately 51), *vhh-1* expression was also detected in the posterior mesenchyme

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of the hindlimb buds and in various regions of the brain, including the floor plate and hypothalamic areas (not shown). All panels show lateral views with dorsal side up and anterior end to the left.

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**Figure 18O**

Expression of HNF-3 $\beta$  protein in a tadpole (stage approximately 36) stage embryo. The expression of HNF-3 $\beta$  is nuclear. Within the central nervous system, cells that express HNF-3 $\beta$  are found in the floor plate (fp) at the ventral midline of the midbrain (m), hindbrain and spinal cord. HNF-3 $\beta$  is not expressed in the ventral region of the rostral diencephalon (d), or in the telencephalon (t). However, expression of HNF-3 $\beta$  as that of *vhh-1* (L, M) and *F-spondin* (Ruiz i Altaba et al., 1993a), is detected in more lateral cells with large nuclei, possibly neurons, in the posterior diencephalon (unlabelled arrows in O). HNF-3 $\beta$  is also expressed in anterior endodermal cells lining the gill and foregut cavities and in posterior endodermal cells at lower levels (not shown). Expression of HNF-3 $\beta$  protein and mRNA (Ruiz i Altaba et al., 1993b) are coincident. Numbers refer to rhombomeres. Rhombomere 4 is located adjacent to the otic vesicle. The panel shows a lateral view with dorsal side up and anterior end to the left.

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**Figure 18N and 18P**

(N, P) Histological sections of tadpole (stage approximately 36) stage embryos showing the expression of *vhh-1* (N) and HNF-3 $\beta$  (P) in the floor plate (fp) of the spinal cord (sc). *vhh-1*, but not HNF-3 $\beta$ , is also expressed at high levels throughout the notochord (n). Cells expressing HNF-3 $\beta$  are detected in the floor plate and in the immediately adjacent ventral ventricular zone (P, see also Ruiz i Altaba et al., 1993a, b), a region

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that does not express other floor plate markers such as *vhh-1* (N) or *F-spondin* (Ruiz i Altaba et al., 1993a). Within the hindbrain, the expression of HNF-3 $\beta$  shows pronounced rhombomeric variations. HNF-3 $\beta$  in rhombomeres 3 and 5 is expressed exclusively in floor plate cells whereas in rhombomeres 2, 4 and 6 expression extends to adjacent ventricular cells (O and not shown). The appearance of these non-floor plate cells expressing HNF-3 $\beta$  may occur after the competence of neural tube cells to become floor plate is lost. Dorsal side is up.

**Figure 18Q**

(Q) Expression of *vhh-1* in a tadpole stage (stage approximately 36) exogastrulae. In complete exogastrulae *vhh-1* mRNA is expressed in the notochord (n) and prechordal plate at early stages (not shown) and in the notochord and anterior endoderm, including the gill endoderm (ge) at later stages. Expression is also detected in the hypochord (not shown). In no case was expression of *vhh-1* detected in the ectodermal sac containing the neural ectoderm (ne). This panel shows a lateral view with the anterior end of both the ectoderm and endomesoderm to the right. In situ hybridization with sense *vhh-1* RNA probes resulted in the absence of any specific labelling (not shown). Scale bar = 500 $\mu$ m for A-C, E, M, ); 450 $\mu$ m for D, J, L; 80 $\mu$ m for F-I, 300 $\mu$ m for K, N; 150 $\mu$ m for P and 70 $\mu$ m for Q.

**Figure 19A, 19B, 19C**

Widespread ectopic expression of *vhh-1* and HNF-3 $\beta$  from injected plasmids.

(A-C) Expression of *vhh-1* mRNA from injected *vhh-1* plasmids (see Methods). A) In frog embryos injected with frog *vhh-1* and analyzed at early gastrula (stage

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approximately 11.5) stage, ectopic *vhh-1* mRNA is detected at high levels in large patches in dorsal (d) ectodermal cells. B) Similarly, rat *vhh-1* mRNA expression after injection of rat *vhh-1* plasmids is detected in neural ectoderm (arrows) in late gastrula-early neurula stage (stage approximately 12.5-15) embryos. At tadpole (stage approximately 38) stages, rat *vhh-1* mRNA is detected in a mosaic manner (C).

10 **Figure 19D, 19E, 19F**

Expression of HNF-3 $\beta$  protein after injection of HNF-3 $\beta$  plasmid.

15 (D) Expression of nucleic HNF-3 $\beta$  protein in large patches of neural and non-neural ectoderm in gastrula (stage approximately 12) stage embryos.

20 (E) Histological section through the dorsal tissues of gastrula stage embryos as that in (D) showing that predominant localization of labelled cells (arrows) in the ectoderm. Expression in the underlying mesoderm is confined to scattered single cells. The endogenous HNF-3 $\beta$  gene is not transcribed in mesodermal or ectodermal cells at these stages (Ruiz i Altaba et al., 1993b).

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(F) At tadpole (stage approximately 36) stages, HNF-3 $\beta$  protein is detected in a mosaic pattern similar to that observed for *vhh-1* in addition to expression of the endogenous gene in the endoderm and the floor plate (fp). However, HNF-3 $\beta$  expression is often detected in the dorsal hindbrain (dh) at high levels (Table 6). One possible explanation for this may be the activation of the endogenous HNF-3 $\beta$  gene in the dorsal neural tube by plasmid-driven HNF-3 $\beta$  (see Text) Arrows point to regions of expression. v: ventral A,C,F) show lateral views with

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anterior end up (A) or to the left (C, F). (D) shows a dorsal view with anterior end up. In most embryos in (B) and in the section shown in (E) dorsal side is up. Scale bar = 680  $\mu$ m for A, D, F; 1.5mm for B; 450  $\mu$ m for C; 100  $\mu$ m for E.

**Figure 20A, 20B, 20C**

Widespread expression of *vhh-1* induces the ectopic expression of HNF-3 $\beta$

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(A-C) Lateral views of the brain of injected tadpole (stages approximately 28, A and approximately 36, B, C) stage embryos labelled with anti-HNF-3 $\beta$  antibodies. The endogenous expression of HNF-3 $\beta$  is detected in the floor plate (fp). Numbers refer to rhombomeres identified by the presence of boundaries under Nomarski optics and the variation of the ventral domain of HNF-3 $\beta$  expression (see Fig. 180). Restrictions in ectopic floor plate marker expression were also found within the hindbrain. A comparison of the location of HNF-3 $\beta$  cells in relation to morphologically visible rhombomeric boundaries revealed preferential ectopic expression in the dorsal region of rhombomere 4, located opposite the otic vesicle, but not in the adjacent rhombomeres 3 and 5. A bias in the ectopic expression of HNF-3 $\beta$  in even versus odd rhombomeres is consistent with evidence that these two rhombomeres display properties not shared by even numbered rhombomeres (Lumsden and Keynes, 1989; Bradley, et al., 1992; Winning and Sargent, 1994).

30

**Figure 20D, 20E and 20F**

(D, E, F) Histological sections of embryos comparable to those in (B, C) showing expression of endogenous HNF-3 $\beta$  protein in the floor plate (fp) overlying the notochord (n) and in adjacent cells and ectopic expression

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restricted to dorsal midline regions including the roof plate (rp) (E, E) and adjacent dorsal alar plate region (arrow in D). A branched neurocoel (bne) is often detected associated with ectopic HNF-3 $\beta$  expression in dorsal cells (E). Ectopic expression is also detected in the otic vesicle (ov) and rarely in cells outside of the neural tube in between the otic vesicle and the dorsal neural tube (F). Within the otic vesicle, highest expression is detected in dorsal regions at late tadpole stages whereas at earlier stages, expression is uniform throughout the otic placode. (A-C) show lateral views with anterior end to the left and in (D-F) dorsal side is up. Cells in the otic vesicle express ectopic (HNF-3 $\beta$  but not *vhh-1* and cells in the epidermis express ectopic *vhh-1* but not HNF-3 $\beta$  (D, F and not shown). This suggests that aspects of the molecular interactions between vertebrate *hedgehog* and winged-helix genes are present in non-neural tissues. Arrowheads point to the sites of ectopic expression. Scale bar = 400  $\mu$ m for A, B; 200  $\mu$ m for C; 75  $\mu$ m for D. E; 100  $\mu$ m for F.

**Figure 21A**

Widespread expression of rat *vhh-1* induces the ectopic expression of *frag vhh-1*

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(A) Expression of frog *vhh-1* at the late gastrula (stage approximately 13) stage after injection of rat-*vhh-1* plasmid. Endogenous expression is detected in the notochord (n) anterior to the blastopore (bp). Ectopic expression is also detected in a few scattered cells (see text).

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**Figure 21B and 21C**

(B, C) Expression of frog *vhh-1* in tadpole (stage approximately 36) stage embryos after widespread

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expression of rat *vhh-1*. In addition to the endogenous expression in the floor plate (fp) and notochord (n), ectopic expression is detected in dorsal regions in the hindbrain and spinal cord (B, C) and in a continuous D-V stripe in the anterior spinal cord (B). Sites of expression along the entire D-V extent of the neural tube were detected only in embryos showing one or more dorsally restricted ectopic expression sites.

10 **Figure 21D, 21E and 21F**

(D-F) Histological sections of the neural tube of tadpole stage embryos comparable to those in (B, C) showing the normal expression of *vhh-1* in the floor plate (fp) and the dorsal restriction of ectopic *vhh-1* expression (D, F) and expression in a medial septum in embryos showing extreme malformations (E). These defects are more prominent at tailbud than at tadpole stages. Branched neurocoels (bne) are often associated with ectopic *vhh-1* expression in dorsal midline regions (F). The dorsal ectopic expression of frog *vhh-1* detected after injection of rat *vhh-1* is unlikely to reflect cross-hybridization with residual plasmid-derived rat *vhh-1* mRNA since this would not be expected to be dorsally restricted. (A) shows a dorsal view with anterior end to the upper left side. B, C) show lateral views with anterior end to the left. In (D, F) dorsal side is up. Arrowheads point to the sites of ectopic expression. Scale bar = 600  $\mu$ m for A-C; 75  $\mu$ m for D-F.

30 **Figure 22A and 22B**

Widespread expression of HNF-3 $\beta$  induces the ectopic expression of *vhh-1* and *F-Spondin*.

(A, B) Expression of *vhh-1* mRNA in tadpole (stage approximately 36) stage embryos injected with HNF-3 $\beta$

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plasmids. Endogenous expression is detected in the floor plate (fp), notochord (n), diencephalon (d) and anterior endoderm. Ecotopic expression is detected in dorsal hindbrain, midbrain and diencephalic regions (A) and in the dorsal spinal cord (B). Analysis of the restriction of ectopic *vhh-1* expression along the A-P axis of the hindbrain was not carried out because it was difficult to distinguish rhombomere boundaries after processing embryos for in situ hybridization.

**Figure 22C**

(C) Histological section of a tadpole (stage approximately 36) stage embryo injected with HNF-3 $\beta$  plasmids, similar to that shown in Figure 19F, displaying expression of HNF-3 $\beta$  protein in the dorsal neural tube. Endogenous expression is detected in the nuclei of floor plate (fp) cells.

**Figure 22D**

(D) Histological section through the diencephalon (d) of a tadpole (stage approximately 36) stage embryo similar to that shown in (A) displaying endogenous expression of *vhh-1* in the ventricular zone of the ventral diencephalon. Ecotopic expression is detected in dorsal ventricular cells.

**Figure 22E and 22F**

(E, F) Expression of *F-spondin* in the floor plate (fp) of normal tadpole (stage approximately 36) embryos (E) and in a sibling embryo injected with HNF-3 $\beta$  plasmid (F). Ecotopic expression is detected in the dorsal ventricular zone. ov: otic vesicle. A, B) show lateral views with anterior end to the left. In (C-F) dorsal side is up. Arrowheads point to the sites of ectopic expression. Scale bar = 580  $\mu$ m for A; 1 mm for B; 75  $\mu$ m for C-F.

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**Figure 23A**

Summary of the normal and ectopic expression of floor plate markers, and the molecular interactions implicated in floor plate differentiation.

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(A) Summary of the normal expression of *Pintallavis* and *vhh-1* at neural plate stages (left) and of HNF-3 $\beta$ , *vhh-1* and *F-spondin* at neural tube stages (right). Note the normal restriction of floor plate marker expression to the midline.

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**Figure 23B**

(B) Summary of the expression of *Pintallavis*, HNF-3 $\beta$ , and *vhh-1* at neural plate stages (left) and of HNF-3 $\beta$ , *vhh-1* and *F-spondin* at neural tube stages (right) in injected embryos. Ectopic expression is induced by widespread expression of HNF-3 $\beta$  or *vhh-1* and detected preferentially in dorsal regions and in the ventricular zone at neural tube stages. See text and Table 6 for other details.

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**Figure 23C**

(C) Summary of the ability (+) or inability (-) of neural cells in the neural plate (left) and neural tube (right) to response to widespread expression of *vhh-1* or HNF-3 $\beta$ .

25

**Figure 23D**

(D) Proposed molecular interactions involved in the induction and differentiation of floor plate cells. Intercellular signalling mediated by *vhh-1* is depicted by arrows with unfilled heads. Intracellular interactions mediated by winged-helix transcription factors are depicted by filled arrows. The limits on the spread of floor plate differentiation through the neural plate by homeogenetic induction are shown by interrupted dashed arrows. See text for details.

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**Figure 24**

Schematic diagram of a cross section through the hindbrain of a tadpole stage embryo (stage approximately 36) showing the different zones which localize ectopic floor plate marker expression in (A). The different regions shown are also representative of the midbrain and spinal cord but all sites located in the dorsal alar plate were scored in the hindbrain. Note that in all cases the roof plate is the major site of expression even though this region contains a small proportion of cells in the neural tube. The basis for the variations in the incidence of ectopic *vhh-1* and *HNF-3 $\beta$*  in different regions (e.g. DAP versus VZ) is not clear. It is possible that expression of injected plasmids in the dorsal ectoderm differentially affects neighboring neural tube (RP and DAP) cells. Ectodermal cells expressing *vhh-1* but not *HNF-3 $\beta$*  might be expected to affect adjacent neural tube cells since only *vhh-1* can act intercellularly. RP = roof plate, DAP = dorsal alar plate immediately adjacent to the roof plate, AP+BP = alar basal plates minus dorsal most region and alar plate, VZ = ventricular zone, V = ventral region adjacent to the floor plate, FP = floor plate.

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Detailed Description of the Invention

This invention provides an isolated DNA molecule encoding a vertebrate vhh-1 protein. As used herein, the term  
5 "isolated nucleic acid molecule" means a non-naturally occurring nucleic acid molecule that is, a molecule which does not occur in nature. Examples of such an isolated nucleic acid molecule are isolated cDNA or genomic DNA molecules encoding a vertebrate vhh-1 protein. This  
10 invention provides an isolated nucleic acid molecule encoding a vertebrate vhh-1 protein wherein the nucleic acid molecule is a DNA molecule. This invention further provides an isolated DNA molecule encoding a vertebrate vhh-1 protein, wherein the DNA molecule is a cDNA  
15 molecule.

In an embodiment, the nucleic acid molecule encodes a frog vhh-1 protein. In another embodiment, the nucleic acid molecule encodes a mammalian vhh-1 protein.

20 A preferred embodiment of a nucleic acid encoding a vertebrate vhh-1 protein is a nucleic acid molecule encoding the rat vhh-1 protein. Such a molecule may have coding sequences the same or substantially the same as  
25 the coding sequences shown in Figures 1-1, 1-2 and 1-3 (Seq I.D. No. 1).

Another preferred embodiment of an isolated nucleic acid molecule encoding a vertebrate vhh-1 protein is a nucleic  
30 acid molecule encoding the human vhh-1 protein. This invention provides an isolated nucleic acid molecule encoding a vertebrate vhh-1 protein, wherein the isolated nucleic acid molecule encodes a human vhh-1 protein.

35 This invention further provides an isolated nucleic acid

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molecule encoding the human vhh-1 protein, wherein the nucleic acid molecule is DNA.

One means of isolating a vertebrate vhh-1 protein is to probe a mammalian genomic library with a natural or artificially designed DNA probe, using methods well known in the art. In one embodiment of this invention, the rat vhh-1 protein and the nucleic acid molecules encoding them are isolated from a rat cDNA library. DNA and cDNA molecules which encode rat vhh-1 protein are used to obtain complementary genomic DNA, cDNA or RNA from human, mammalian or other animal sources, or to isolate related cDNA or genomic clones by the screening of cDNA or genomic libraries, by methods described in more detail below. Transcriptional regulatory elements from the 5' untranslated region of the isolated clone, and other stability, processing, transcription, translation, and tissue specificity determining regions from the 3' and 5' untranslated regions of the isolated gene are thereby obtained.

The human homolog of the rat vhh-1 gene is isolated using the rat vhh-1 probe described hereinabove and cloning techniques known to one of skill in the art, such as homology screening of genomic or cDNA libraries or PCR amplification techniques. The vhh-1 gene is expressed in the lungs of older embryos, therefore the preferred method of cloning the human vhh-1 gene involves screening the clontech human fetal lung cDNA library to obtain the human clone. The rat vhh-1 has been used to identify the chick and frog vhh-1 genes (see below for the frog gene data) and will therefore be sufficiently conserved to identify the human vhh-1 gene.

This invention provides a vector comprising a nucleic

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acid molecule encoding a vertebrate vhh-1 protein. Examples of vectors are viruses such as bacteriophages (including but not limited to phage lambda), animal viruses (including but not limited to baculovirus, vaccinia virus, Herpes virus, and Murine Leukemia virus), cosmids, plasmids and other recombination vectors are well known in the art. Nucleic acid molecules are inserted into vector genomes by methods well known to those skilled in the art. To obtain these vectors, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules which base pair with each other and are then ligated together with a ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA, which is then digested with the restriction enzyme which cuts at that site. Other means are also known to one of skill in the art.

This invention provides a plasmid comprising the vector comprising an isolated nucleic acid molecule encoding a vertebrate vhh-1 protein. Examples of such plasmids are plasmids comprising cDNA having a coding sequence the same or substantially the same as: the coding sequence shown in Figures 1-1, 1-2 and 1-3 (Seq. I.D. No. 1) and designated clone pMT21 2hh #7 deposited under ATCC Accession No. 75686 and designated clone cmv vhh #7 deposited under ATCC Accession No. 75685.

Expression vectors can be adapted for expression in a bacterial cell, a yeast cell, an insect cell, a *Xenopus* oocyte or a mammalian cell which additionally are operatively linked to regulatory elements necessary for expression of the inserted gene in the bacterial, yeast, insect, frog or mammalian cells. DNA having coding

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sequences substantially the same as the coding sequence shown in Figures 1-1, 1-2 and 1-3 can be inserted into the vectors for expression using the methods discussed hereinabove or other methods known to one of skill in the art. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome operatively linked to the recombinant gene. Furthermore, an insect expression vector such as baculovirus AcMNPV uses the strong viral expression signals for the virus' polyhedron gene to drive transcription of the recombinant gene. One such example of a plasmid comprising regulatory elements for expression in oocytes operatively linked to the recombinant vhh-1 gene is the plasmid designated cmv vhh #7 and deposited under ATCC Accession No. 75685. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that express the vhh-1 protein. Certain uses for such cells are described in more detail below.

Deposits were made on February 24, 1994 of both the pMT21 2hh #7 and cmv vhh #7 plasmids with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852. The two deposits were made pursuant to, and in satisfaction of, the provisions of



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the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure with the ATCC.

5 Plasmid, pMT21 vhh #7, is produced by cloning a 2.6 kilobase fragment of the rat vhh-1 gene which contains the complete coding region and both 3' and 5' untranslated regions into the XhoI site of the plasmid pMT 21. The 2.6 kilobase can be regenerated by XhoI  
10 digestion.

Plasmid cmv vhh #7 also contains the 2.6 kilobase fragment of the rat vhh-1 gene which has the complete coding region and both 3' and 5' untranslated regions.  
15 The 2.6 kilobase XhoI insert is cloned into the SalI site such that the XhoI sites are destroyed. The insert is under the control of an upstream CMV promoter and further upstream by a Hox 2.6 enhancer. Downstream from the insert is a 0.8 kilobase poly A site of SV40 and then  
20 linked to a hygromycin gene (PGK HYG). NotI digest will linearize the plasmid.

This invention provides a mammalian cell comprising an expression plasmid encoding a vertebrate vhh-1 protein.  
25 This invention also provides a mammalian cell comprising an expression plasmid encoding a mammalian vhh-1 protein. This invention further provides a Cos cell comprising an expression plasmid encoding a vertebrate vhh-1 protein.

30 Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell NIH3T3, CHO cells, HeLa cells, Cos cells, and 293 cells. Expression plasmids such as that described supra may be used to transfect mammalian cells by methods well known in the  
35 art such as calcium phosphate precipitation, or DNA

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encoding the vhh-1 protein may be otherwise introduced into mammalian cells, e.g., by microinjection, to obtain mammalian cells which comprise DNA, e.g., cDNA or a plasmid, encoding a vertebrate vhh-1 protein.

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This invention provides a nucleic acid molecule probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule comprising the gene encoding the vertebrate vhh-1 protein and its noncoding 3' and 5' nucleotides.

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As used herein, the phrase "specifically hybridizing" means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs. As used herein, a "unique sequence" is a sequence specific to only the nucleic acid molecules encoding the vertebrate vhh-1 protein. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe.

Detection of nucleic acid molecules encoding the vertebrate vhh-1 protein is useful as a diagnostic test for any disease process in which levels of expression of the corresponding vhh-1 protein is altered. DNA probe molecules are produced by insertion of a DNA molecule which encodes vertebrate vhh-1 protein or fragments thereof into suitable vectors, such as plasmids or bacteriophages, followed by insertion into suitable bacterial host cells and replication and harvesting of the DNA probes, all using methods well known in the art.

For example, the DNA may be extracted from a cell lysate

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using phenol and ethanol, digested with restriction enzymes corresponding to the insertion sites of the DNA into the vector (discussed above), electrophoresed, and cut out of the resulting gel. Examples of such DNA molecules are shown in Figures 1-1, 1-2 and 1-3. The probes are useful for 'in situ' hybridization or in order to locate tissues which express this gene family, or for other hybridization assays for the presence of these genes or their mRNA in various biological tissues. In addition, synthesized oligonucleotides (produced by a DNA synthesizer) complementary to the sequence of a DNA molecule which encodes a vertebrate vhh-1 protein are useful as probes for this gene, for its associated mRNA, or for the isolation of related genes by homology screening of genomic or cDNA libraries, or by the use of amplification techniques such as the Polymerase Chain Reaction.

A preferred embodiment of a nucleic acid molecule probe of a vertebrate vhh-1 protein is a DNA molecule probe.

This invention provides a purified vertebrate vhh-1 protein. In an embodiment, the purified vhh-1 protein is a frog vhh-1 protein. In another embodiment, the purified vhh-1 protein is a mammalian protein. In a further embodiment, the purified vhh-1 protein is a rat protein. In a still further embodiment, the purified vhh-1 protein is a human protein.

This invention further provides a purified unique polypeptide fragment of the vertebrate vhh-1 protein.

As used herein, the term "unique polypeptide fragment" encompasses any polypeptide with the same amino acid sequence as any unique amino acid sequence as shown in

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Figures 1-1, 1-2 and 1-3 (Sequence ID No. 2). One means for obtaining an isolated polypeptide fragment of a vertebrate vhh-1 protein is to treat isolated vhh-1 protein with commercially available peptidases and then  
5 separate the polypeptide fragments using methods well known to those skilled in the art. Polypeptide fragments are often useful as antigens used to induce an immune response and subsequently generate antibodies against the polypeptide fragment and possibly the whole polypeptide.

10

As used herein, the term "purified protein" is intended to encompass a protein molecule free of other cellular components. One means for obtaining purified vhh-1 protein is to express DNA encoding the vhh-1 protein in  
15 a suitable host, such as a bacterial, yeast, insect, or mammalian cell, using methods well known to those skilled in the art, and recovering the vhh-1 protein after it has been expressed in such a host, again using methods well known in the art. The vhh-1 protein may also be isolated  
20 from cells which express the vhh-1 protein, in particular from cells which have been transfected with the expression vectors described below in more detail.

This invention provides a monoclonal antibody directed to  
25 a vertebrate vhh-1 protein.

This invention further provides a monoclonal antibody, directed to an epitope of a vertebrate vhh-1 protein and having an amino acid sequence substantially the same as  
30 an amino acid sequence for an epitope of a vertebrate vhh-1 protein.

This invention further provides a monoclonal antibody, wherein the monoclonal antibody is directed to the frog  
35 vhh-1 protein.

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This invention further provides a monoclonal antibody, wherein the monoclonal antibody is directed to the rat vhh-1 protein.

- 5 This invention further provides a monoclonal antibody, wherein the monoclonal antibody is directed to the mammalian vhh-1 protein.

10 This invention further provides a monoclonal antibody, wherein the monoclonal antibody is directed to the human vhh-1 protein.

Monoclonal antibody directed to a vertebrate vhh-1 protein may comprise, for example, a monoclonal antibody directed to an epitope of a vertebrate vhh-1 protein present on the surface of a cell, the epitope having an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the vertebrate vhh-1 protein included in the amino acid sequence shown in Figures 1-1, 1-2 and 1-3. Amino acid sequences may be analyzed by methods well known to those skilled in the art to determine whether they produce hydrophobic or hydrophilic regions in the proteins which they build. In the case of cell membrane proteins, hydrophobic regions are well known to form the part of the protein that is inserted into the lipid bilayer which forms the cell membrane, while hydrophilic regions are located on the cell surface, in an aqueous environment. Therefore antibodies to the hydrophilic amino acid sequences shown in Figures 1-1, 1-2 and 1-3 will bind to a surface epitope of a vertebrate vhh-1 protein, as described. Antibodies directed to vertebrate vhh-1 protein may be serum-derived or monoclonal and are prepared using methods well known in the art. For example, monoclonal antibodies are prepared using

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hybridoma technology by fusing antibody producing B cells from immunized animals with myeloma cells and selecting the resulting hybridoma cell line producing the desired antibody. Cells such as NIH3T3 cells or 293 cells may be  
5 used as immunogens to raise such an antibody. Alternatively, synthetic peptides may be prepared using commercially available machines and the amino acid sequences shown in Figures 1-1, 1-2 and 1-3.

10 As a still further alternative, DNA, such as a cDNA or a fragment thereof, may be cloned and expressed and the resulting polypeptide recovered and used as an immunogen. These antibodies are useful to detect the presence of vertebrate vhh-1 encoded by the isolated DNA, or to  
15 inhibit the function of the vhh-1 protein in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

This invention provides polyclonal antibodies directed to  
20 a vertebrate vhh-1 protein.

Animal model systems which elucidate the physiological and behavioral roles of vertebrate vhh-1 protein are produced by creating transgenic animals in which the  
25 expression of a vhh-1 protein is either increased or decreased, or the amino acid sequence of the expressed vhh-1 protein is altered, by a variety of techniques. Examples of these techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA  
30 encoding a rat vhh-1 or homologous animal versions of these genes, especially the human homolog of the vhh-1 gene, by microinjection, retroviral infection or other means well known to those skilled in the art, into appropriate fertilized embryos in order to produce a  
35 transgenic animal (Hogan B. et al. Manipulating the Mouse

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Embryo, A Laboratory Manual, Cold Spring Harbor Laboratory (1986)) or, 2) Homologous recombination (Capecchi M.R. Science 244:1288-1292 (1989); Zimmer, A. and Gruss, P. Nature 338:150-153 (1989)) of mutant or  
5 normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the structure of these vhh-1 proteins. The technique of homologous recombination is well known in the art. It replaces the native gene with  
10 the inserted gene and so is useful for producing an animal that cannot express native gene encoding the vhh-1 protein but does express, for example, an inserted mutant gene encoding a mutant vhh-1 protein, which has replaced the native vhh-1 gene in the animal's genome by  
15 recombination, resulting in underexpression of the vhh-1 protein. Microinjection adds genes to the genome, but does not remove them, and so is useful for producing an animal which expresses its own and added vhh-1 protein, resulting in overexpression of the vhh-1 protein.

20 This invention provides a transgenic nonhuman mammal which comprises an isolated DNA molecule encoding a vertebrate vhh-1 protein.

25 One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium (Hogan B. et al. Manipulating the Mouse Embryo, A Laboratory Manual, Cold  
30 Spring Harbor Laboratory (1986)). DNA or cDNA encoding a vertebrate vhh-1 protein is purified from a vector (such as plasmid pMT21 2hh #7 described above) by methods well known in the art. Inducible promoters may be fused  
35 with the coding region of the DNA to provide an

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experimental means to regulate expression of the trans-gene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene.

5 The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA

10 solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term.

15 As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

Since the normal action of vhh-1 protein-specific drugs is to mimic activate or inhibit the vhh-1 protein, the transgenic animal model systems described above are useful for testing the biological activity of drugs directed to mimic or alter the vhh-1 protein activity even before such drugs become available. These animal

20 model systems are useful for predicting or evaluating possible therapeutic applications of drugs which mimic, activate or inhibit the rat vhh-1 protein by alleviating abnormalities observed in the transgenic animals associated with decreased or increased expression of the native vhh-1 gene or vhh-1 trans-gene. Thus, a model

25 system is produced in which the biological activity of drugs specific for the vhh-1 protein are evaluated before such drugs become available. The transgenic animals which over or under produce the vhh-1 protein indicate by

30 their physiological state whether over or under

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production of the vhh-1 protein is therapeutically useful. It is therefore useful to evaluate drug action based on the transgenic model system. Therefore, an animal which underexpresses vhh-1 protein is useful as a test system to investigate whether the actions of a pharmaceutical compound comprising vhh-1 is in fact therapeutic. Another use is that if overexpression is found to lead to abnormalities, then a drug which acts as an antagonist to the vhh-1 protein is indicated as worth developing, and if a promising therapeutic application is uncovered by these animal model systems, activation or inhibition of the vhh-1 protein is achieved therapeutically either by producing agonist or antagonist drugs directed against the vertebrate vhh-1 protein or by any method which increases or decreases the activity of the vhh-1 protein.

This invention provides a transgenic nonhuman mammal which comprises an isolated DNA molecule encoding a rat vhh-1 protein.

This invention further provides the transgenic nonhuman mammal which comprises an isolated DNA molecule encoding a vertebrate vhh-1 protein, wherein the DNA encoding a vertebrate vhh-1 protein additionally comprises tissue specific regulatory elements.

This invention provides a transgenic nonhuman mammal which comprises the isolated DNA molecule encoding a human vhh-1 protein.

This invention provides a method of determining the physiological effects of expressing varying levels of a vertebrate vhh-1 protein which comprises producing a panel of transgenic nonhuman animals each expressing a

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different amount of vertebrate vhh-1 protein. Such animals may be produced by introducing different amounts of DNA encoding a rat vhh-1 protein into the oocytes from which the transgenic animals are developed.

5

This invention provides a method of producing a purified vertebrate vhh-1 protein which comprises: (a) inserting nucleic acid molecule encoding the vertebrate vhh-1 protein in a suitable vector; (b) introducing the resulting vector in a suitable host cell; (c) selecting the introduced host cell for the expression of the vertebrate vhh-1 protein; (d) culturing the selected cell to produce the vhh-1 protein; and (e) recovering the vhh-1 protein produced.

15

This invention further provides the above-described method to produce purified frog, mammalian, rat and human vhh-1 proteins.

20

These methods for producing vhh-1 proteins involve methods well known in the art. For example, isolated nucleic acid molecule encoding frog, rat or human vhh-1 protein is inserted in a suitable vector, such as an expression vector. A suitable host cell, such as a bacterial cell, or a eukaryotic cell such as a yeast cell, or an insect cell is transfected with the vector. The vertebrate protein is isolated from the culture medium by affinity purification or by chromatography or by other methods well known in the art.

25

This invention provides a method of inducing the differentiation of floor plate cells comprising contacting floor plate cells with a purified vertebrate vhh-1 protein at a concentration effective to induce the differentiation of floor plate cells.

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This invention provides a method of inducing the differentiation of floor plate cells in a subject comprising administering to the subject a purified vertebrate vhh-1 protein at an amount effective to induce the differentiation of floor plate cells in the subject.

This invention provides a method of inducing the differentiation of motor neuron comprising contacting the floor plate cells with a purified vertebrate vhh-1 protein at a concentration effective to induce the differentiation of motor neuron.

This invention provides a method of inducing the differentiation of motor neuron in a subject comprising administering to the subject a purified vertebrate vhh-1 protein at an amount effective to induce the differentiation of motor neuron in the subject.

This invention provides a method of generating ventral neurons comprising contacting progenitor cells with a purified vertebrate vhh-1 protein at a concentration effective to generate ventral neurons.

This invention provides a method of generating ventral neurons from progenitor cells in a subject comprising administering to the subject a purified vertebrate vhh-1 protein at an amount effective to generate ventral neurons from progenitor cells in the subject.

This invention provides a pharmaceutical composition comprising an effective amount of a vertebrate vhh-1 protein and a pharmaceutically acceptable carrier.

This invention provides a pharmaceutical composition comprising an effective amount of a mammalian vhh-1

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protein and a pharmaceutically acceptable carrier.

5 This invention provides a pharmaceutical composition comprising an effective amount of a human vhh-1 protein and a pharmaceutically acceptable carrier.

10 As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. Once the candidate drug has been shown to be adequately bio-available following a particular route of administration, for example orally or by injection (adequate therapeutic  
15 concentrations must be maintained at the site of action for an adequate period to gain the desired therapeutic benefit), and has been shown to be non-toxic and therapeutically effective in appropriate disease models, the drug may be administered to patients by that route of  
20 administration determined to make the drug bio-available, in an appropriate solid or solution formulation, to gain the desired therapeutic benefit.

25 Delivery of pharmaceutical compositions to sites of vhh-1 protein action propose a complex problem. vhh-1 induces nondifferentiated motor neuron precursor cells to differentiate into motor neurons. Since the regeneration of motor neurons for the purpose of alleviating abnormalities associated with acute nervous system injury or chronic neurodegenerative diseases requires  
30 differentiation of motor neuron precursor cells which reside in the central nervous system (CNS), pharmaceutical compounds comprising the vhh-1 protein or drugs or substances that alter vhh-1 protein action must  
35 be delivered into the CNS. vhh-1 does not pass through

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the blood-brain barrier and therefore pharmaceutical compositions comprising same must be given intracerebrally, surgically implanted within the CNS, or complexed to a carrier molecule (such as transferrin) capable of crossing the blood-brain barrier. A neurotrophic factor, NGF, has been chronically infused into the brain by a mechanical pump device which allow consistent delivery of NGF into the CNS (Koliatos et al. 1991 and Olsen et al. 1992). In the case of acute nervous system injury involving specific central axon(s), slow release implants containing vhh-1 in a known biodegradable polymer matrix could be surgically implanted at the site of the injured axon(s) effective to regenerate motor neurons from motor neuron precursor cells proximal to the injured axon. Another neurotrophic factor, NGF, has successfully been implanted in such a manner to prevent degeneration of cholinergic neurons (Hoffman et al. 1990 and Maysinger et al. 1992). Another method of implanting a source of vhh-1 next to an injured axon requires the transfection of cells incapable of proliferation and further encapsulated to avoid infiltration of the CNS wherein such cells comprise a plasmid encoding the human vhh-1 gene and therefore express vhh-1. Aebischer et al. (1991) successfully implanted encapsulated growth factor producing cells to avoid infiltration of brain tissue. Neurotrophic factors have successfully been conjugated to carrier molecules that shuttle the factor into the CNS. One such example is NGF which has been conjugated to a carrier molecule, monoclonal anti-transferrin receptor antibodies, effective to deliver the neurotrophic factor into the CNS (Friden et al. 1993).

This invention provides a method for treating a human subject afflicted with an abnormality associated with the

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lack of one or more normally functioning motor neuron(s) which comprises introducing an amount of a pharmaceutical composition comprising an amount of a human vhh-1 protein and a pharmaceutically acceptable carrier effective to  
5 generate motor neurons from undifferentiated motor neuron precursor cells in a human, thereby treating a human subject afflicted with an abnormality associated with a lack of one or more normally functioning motor neuron(s).

10 This invention provides a method for treating a human subject afflicted with an abnormality associated with the lack of one or more normally functioning motor neuron(s) which comprises introducing an amount of a pharmaceutical composition comprising an amount of a human vhh-1 protein  
15 and a pharmaceutically acceptable carrier effective to generate motor neurons from undifferentiated motor neuron precursor cells in a human, thereby treating a human subject afflicted with an abnormality associated with a lack of one or more normally functioning motor neuron(s),

20 This invention provides a method of treating a human subject afflicted with a neurodegenerative disease which comprises introducing an amount of a pharmaceutical composition comprising an amount of a human vhh-1 protein  
25 and a pharmaceutically acceptable carrier effective to generate motor neurons from undifferentiated motor neuron precursor cells in a human, thereby treating a human subject afflicted with a neurodegenerative disease.

30 This invention provides a method of treating a human subject afflicted with a neurodegenerative disease, wherein the chronic neurodegenerative disease is Amyotrophic lateral sclerosis (ALS), which comprises introducing an amount of a pharmaceutical composition  
35 comprising an amount of a human vhh-1 protein and a

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pharmaceutically acceptable carrier effective to generate motor neurons from undifferentiated motor neuron precursor cells in a human, thereby treating a human subject afflicted with Amyotrophic lateral sclerosis (ALS).

A method of treating a human subject afflicted with an acute nervous system injury which comprises introducing an amount of a pharmaceutical composition comprising an amount of a human vhh-1 protein and a pharmaceutically acceptable carrier effective to generate motor neurons from undifferentiated motor neuron precursor cells in a human, thereby treating a human subject afflicted with an acute nervous system injury.

A method of treating a human subject afflicted with an acute nervous system injury, wherein an acute nervous system injury is localized to a specific central axon which comprises surgical implantation of an amount of a pharmaceutical composition comprising the human vhh-1 protein and a pharmaceutically acceptable carrier effective to generate motor neurons from undifferentiated motor neuron precursor cells located proximal to the injured axon in a human, thereby alleviating an acute nervous system injury localized to a specific central axon.

Elucidation of the molecular structures of the neurotrophic factor designated as the vhh-1 protein is an important step in the understanding of new neurotrophic factors. This disclosure reports the isolation, amino acid sequence, and functional expression of a cDNA clone from rat brain which encodes a vhh-1 protein. Analysis of the rat vhh-1 protein structure and function provides a possible model for the development of drugs useful for

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the treatment of acute nervous system injury or chronic neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS).

5 Specifically, this invention relates to the first isolation of a cDNA clone encoding a rat vhh-1 protein. The vertebrate vhh-1 gene is expressed in restricted regions of the embryo, in particular the notochord and floor plate, two cell groups which have been shown to  
10 induce ventral cell types including the floor plate and motor neurons. The vertebrate gene for this vhh-1 protein has been characterized in vivo and in vitro to elucidate the role of vhh-1 in inducing the developmental differentiation of motor neurons and floor plate in  
15 embryos. The vhh-1 protein is likely to be useful in the treatment of degenerative disorders of the central nervous system, in particular motor neuron degeneration, and this may be useful in the treatment of a number of clinical disorders that result in motor dysfunction. In  
20 addition, the rat vhh-1 protein has been expressed in COS cells by transfecting the cells with the plasmid pMT21 2hh #7.

25 The invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative, and are not meant to limit the invention as described herein, which is defined by the claims which follow thereafter.



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EXPERIMENTAL DETAILSAnimals

5 Zebrafish embryos were obtained from the colony at the  
Department of Microbiology, Umea University, Sweden,  
Pregnant female rats (Hilltop) were delivered by  
Caesarean section and embryos staged according to somite  
10 from SPAFAS, Incorporated (Norwich, Connecticut). chick  
embryos were staged according to Hamburger and Hamilton  
(1951). Frog (*Xenopus laevis*) eggs and embryos were  
reared and staged according to Nieuwkoop and Faber (1957)  
and Ruiz i Altaba (1993).

15

Isolation of Vertebrate Genes Related to *hh*

Plaques (10<sup>4</sup>) of a 9-16 hr. postfertilization  $\lambda$ ZAPII  
zebrafish library were screened at low stringency with  
20 *Drosophila hh* cDNA (provided by J. Mohler) and with DNA  
fragments generated by polymerase chain reaction using  
the *hh* sequence (Lee et al., 1992) as a template. Two  
sets of polymerase chain reaction primers were used 5'-  
GAGGATTGGGTCGTCATAGG-3' (positions 652-671 in the  
25 *Drosophila hh* cDNA) and 5'-CTTCAAGGATTCCATCTCAA-3'  
(positions 1799-1818); 5'AGCTGGGACGAGGACTACCATC-3'  
(positions 945-966) and 5'TGGGAACTGATCGACGAATCTG-3'  
(positions 1147-1128). Clones isolated with the second  
primer set were subcloned and sequenced on both strands  
30 by the dideoxy chain termination method (Sanger et al.,  
1977). DNA and derived amino acid sequences were  
analyzed on a VAX computer using the Genus software  
package.

35 To identify rat *hh*-related cDNA clones, approximately 2.5

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x 10<sup>5</sup> colonies of a rat E13 floor plaque cDNA library in pMT21 were screened with the zebrafish *vhh-1* probe in HM mix (5 x Denhardt's solution, 10% dextran sulphate, 2x SSC, 2x SSPE, 0.5% SDS, and 50 µg/ml denatured herring sperm DNA) at 60°C. XhoI cDNA inserts from hybridizing clones were subcloned in pBluscript II KS(-) and sequenced on both strands by the dideoxy chain termination methods (Sanger et al., 1977). Sequence analysis and compilations were performed on a VAX computer using GCG software.

#### In Situ Hybridization

Whole-mount in situ hybridization analysis of mRNA expression were performed with digoxigenin-labeled probes essentially as described by Harland (1991) and Krauss et al. (1991) with minor modifications (Ruiz i Altaba et al., 1993b) and for cryostat sections as described by Schaeren-Wiemers and Gerfin-Moser (1993). For each species, the probe used included coding and noncoding regions. Control hybridizations contained sense strand probes or antisense probes directed against other genes. The frog F-spondin gene (Ruiz i Altaba et al., 1993b) was transcribed with T7 RNA polymerase after digestion with HindIII) to generate an antisense probe.

#### Expression of *vhh-1* in COS cells

Cos cells were grown overnight until 90% confluent and transfected with 1 µg of DNA per 35 mm dish with 12 µg/ml lipofectamino reagent (GIBCO BRL) in Dulbeccos' modified Eagle's medium (DMEM). After 5 hours, cells were washed and incubated in DMEM containing 10% FCS for 18 hours. The medium was then replaced by fresh DMEM containing 10% FCS and cells were incubated for 24-48 hours. COS cells

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were dissociated 24 hours after transfection with enzyme-free dissociation medium (Specially Media, Incorporated), peeled, and resuspended in OptiMEM containing 10% FCS. Aggregates were made by hanging a 20  $\mu$ l drop containing 200-400 cells from the lid of a tissue culture plate. After 24 hours, cell aggregates were placed in contact with rat neural plate explants.

#### Neural Plate Explant Cultures

Rat neural plate tissue was isolated from the intermediate and dorsal regions of the neural plate of E9-E10 embryos (at the level of prospective somites 15-19) as described by Placzek et al. (1990a, 1993). Chick neural plate tissue was dissected from Hamburger-Hamilton stage 10 chick embryos as described (Yamada et al., 1993). Notochord explants were isolated by dissection from stage 6 chick embryos after dispose treatment. Rat neural plate explants were embedded within three-dimensional collagen gels and culture as described (Tessier-Lavigne et al., 1988; Placzek, et al., 1993). Conjugates were made by wrapping the neural plate explants around COS cell aggregates to maximize the extent of contact.

Chick intermediate neural plate explants, about one-third the size of those used by Yamada et al., (1993), were placed on a monolayer of control or transfected COS cells grown for 44 hours in 35 mm tissue culture dishes. A cushion of collagen gel was placed on top of the explant to maintain the position of the explant and the contact with COS cells and cultures were incubated for 44 hours as described (Yamada et al., 1993).

#### Limb Bud Explant Cultures

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Chick limb bud tissue was dissected from Hamburger-Hamilton stage 20 embryos. Mesenchymal tissue that corresponds to the region that expressed *shh* (Riddle et al., 1993) and defined to have ZPA activity (Honig and Summerball, 1985) and adjacent ectoderm was dissected from posterior limb tissue. Similar sized explants were dissected from anterior limb tissue. Explants were treated as described (Placzek et al., 1993). Rat tissues were wedged between mesenchymal and ectodermal layers of the limb bud explants or were opposed to the mesenchymal layer.

#### Expression of *vhh-1* in Frog Embryos

*X. laevis* embryos at the 1-or-2-cell stage were injected with 100-200 pg of supercoiled plasmid DNA. In all cases injections were performed in the animal hemisphere that is fated to give rise to ectodermal derivatives, including the nervous system (Dale and Slack, 1987). Expression of the *vhh-1* cDNA in the sense or antisense orientation in the injected plasmids was driven by the CMV promoter containing the Hox-B4 region A enhancer element (Whitnig et al., 1991). The region A element does not affect the tissue specificity or the level of expression of downstream genes (A.R.A., H.R., AND T.M.J., unpublished data). Expression of *vhh-1* transcripts from the injected plasmids was monitored by whole-mount in situ hybridization using an antisense RNA probe.

#### Immunocytochemistry

Rabbit antibodies against the frog HNF-38 protein were used at 1:5000 to 1:8000 dilution for whole-mount labelling (Dent et al., 1989; Patel et al., 1989). FP3 was detected using monoclonal antibody (MAb) 6G3 (mouse IgG)

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and FP4 was detected using MAb K1/2E7 (mouse IgG1; Placzek et al., 1993). Islet-1 was detected using rabbit anti-islet-1 antibodies diluted 1:1000 (Thor et al., 1991; Korzh et al., 1993) and MAb 4D5 (mouse IgG, raised by S. Morton against a rat islet-1 fusion protein; Thore et al., 1991). The SC1 protein was detected with a MAb provided by H. Tanaka. For identification of FP3 and FP4 in the same explants, serial sections were labeled with antibodies to FP3 and FP4.

10

#### EXPERIMENTAL RESULTS

##### Isolation and Characterization of Vertebrate Homologs of the Drosophila *hh* Gene

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To isolate vertebrate homologs of the Drosophila *hh* gene, zebrafish and rat embryo cDNA libraries were screened with polymerase chain reaction fragments derived from the Drosophila *hh* cDNA. Five clones isolated from a 9-16 hr postfertilization zebrafish embryo library encoded two distinct *hh*-related cDNAs, one of which, *vhh-1*, is described here. The longest *vhh-1* cDNA contained a 2.6 kb insert with a single long open reading frame that encodes a protein of 418 amino acids (Figure 2A-1 and 2A-2). Zebrafish *vhh-1* mRNA expression was confined primarily to midline structures, in particular, the notochord and floor plate. The zebrafish *vhh-1* cDNA was used to screen an embryonic day 13 (E13) rat floor plate cDNA library. Sixteen independent cDNA clones were isolated with inserts ranging in size from 0.8 to 2.7 kb. Partial sequencing of each of these cDNA clones revealed that they derived from the same gene. Sequencing of one 2.7 kb clone revealed a single long open reading frame that predicts a protein of 437 amino acids.

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The rat *vhh-1* cDNA encodes a protein with 71% identity to the zebrafish *vhh-1* protein, 94% identity to mouse *shh* (Echelard et al., 1993), 82% identity to chick *shh* (Riddle et al., 1993), and 47% identity to *Drosophila* *hh* (Figures 2A-1 and 2A-2). The sequence of the zebrafish *shh* (Krauss et al., 1993) with the exception of a region at its COOH-terminal end over residues 437-466 (residues aligned to the fly *hh* sequence; see Figures 2A-1 and 2A-2). Zebrafish *vhh-1* is identical in the region of divergence to the *zhHE* protein isolated by Beachy and colleagues (P. Beachy, personal communication). The greatest degree of conservation between the vertebrate and fly proteins occurs over the NH<sub>2</sub>-terminal 200 amino acids. Both zebrafish and rat *vhh-1* proteins contain a hydrophobic NH<sub>2</sub>-terminus that is likely to serve as a signal sequence (Figure 2B), suggesting that the processed protein is secreted. The similarity in sequence and expression pattern (see below) of the zebrafish and rat *vhh-1* genes and the mouse and chick *shh* genes suggests that they are homologs.

#### Expression of the *vhh-1* Gene during Embryogenesis

The patterns of expression of the zebrafish and rat *vhh-1* genes are similar, and applicants report here only the expression of the rat gene. Applicants first assayed *vhh-1* mRNA expression in gastrulating rat embryos at E9. At this time *vhh-1* mRNA was found in the node and in axial mesodermal cells laid down in the wake of the regressing node (Figure 3A). *vhh-1* mRNA expression persists in midline mesodermal cells as they differentiate into the notochord (Figures 3B and 3C) and is detectable in this structure until E15, the latest stage examined (Figures 3D and 3E). Cells of the neural plate and newly closed neural tube do not express *vhh-1*

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mRNA (Figures 3C and 3D). However, floor plate cells at the rostral region of the spinal cord expressed the gene by E10.5 (Figure 3B), and soon after *vhh-1* mRNA was detectable in the floor plate at all rostrocaudal levels, persisting until at least E 15 (Figure 3E). In the spinal chord and hindbrain, *vhh-1* mRNA expression was restricted to the floor plate as assessed by comparison with other rat floor plate markers (data not shown, Placzek et al., 1993; Ruiz i Altaba et al., 1993b). In the forebrain, *vhh-1* expression is also located more laterally in the ventral diencephalon and is absent from the ventral midline at the level of the infundibulum (data not shown). Within the diencephalon, *vhh-1* mRNA expression extends dorsally up to the boundary between the ventral and dorsal thalamus (data not shown). In the rostral diencephalon, *vhh-1* expression is detected ventrally in the region of the developing hypothalamus. The sole dorsal site of neural expression of *vhh-1* mRNA is a group of cells at the roof of the midbrain that is first detectable at E10.5 (Figure 3B).

*Vhh-1* mRNA was detected in two additional regions of rat embryos from E10.5 to E15. Endodermal cells located in the ventral half of the early gut tube expressed *vhh-1* mRNA (Figure 3B). The intensity of expression of the gene in endodermal derived tissues increases at later stages of development, and by E15-E15 it is expressed at high levels in gut and lung epithelia (data now shown). *vhh-1* mRNA was also expressed in posterior mesenchymal cells of the developing limb bud at E11-E14 (see Figure 7A), which corresponds to the region defined as the zone of polarizing activity (ZPA).

The expression of *vhh-1* in the node, notochord, and floor plate, cell groups with floor plate inducing activity,

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raises the possibility that this gene encodes a floor plate-inducing activity, raises the possibility that this gene encodes a floor plate-inducing molecule. In the following sections we describe the effects of *vhh-1* on the differentiation of ventral neural cell types in vivo and in vitro.

Ectopic Expression of the *vhh-1* Gene in Frog Embryos Leads to Floor Plate Differentiation in the Dorsal Neural Tube

Applicants monitored the consequences of ectopic expression of the *vhh-1* gene in developing frog embryos. Ectopic expression of *vhh-1* was achieved by injecting a plasmid vector containing the rat *vhh-1* cDNA under the control of a cytomegalovirus (CMV) promoter. At neural plate stages (stages 13-17), rat *vhh-1* mRNA was expressed in large patches of cells located primarily in the region of the anterior epidermis and neural plate (11 of 11 embryos examined) (Figures 4A). By the tadpole stage (stages 32-38), however, *vhh-1* mRNA was mosaic and detected in smaller groups of cells (data not shown). Of injected embryos, 31% (23 of 74 examined) showed ectopic expression of *vhh-1* in the neural tube. Within the neural plate and neural tube, there was no consistent restriction in the domain of neural expression of the CMV-driven rat *vhh-1* gene (Figure 4A; data not shown).

Applicants determined whether the widespread expression of *vhh-1* RNA leads to the differentiation of floor plate cells in ectopic locations by monitoring the expression of two floor plate markers, the cell adhesion molecule F-spondin (Klar et al., 1992; Ruiz l Altaba et al., 1993a) (Figures 4B and 4D) and the transcription factor HNF-3 $\beta$  (19 of 153) were detected in regions other than the floor



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plate (Figures 4C, 4E, 4F, 4H and 4I). Ectopic expression of both markers was detected at midbrain, hindbrain, and spinal cord levels but not in forebrain regions (Figures 4E, 4F, 4H, and 4I). Embryos injected with a plasmid driving expression of *vhh-1* cDNA in the antisense orientation showed a markedly lower incidence of ectopic F-spondin expression (2%; 4 of 198), and ectopic HNF-3 $\beta$  cells were not detected (0 of 53). Thus, the widespread expression of rat *vhh-1* in developing frog embryos leads to the ectopic induction of floor plate marker. Although the ectopic expression of HNF-3 $\beta$  and F-spondin RNA was observed at all rostrocaudal levels of the neuraxis except the forebrain, the predominant location of ectopic markers expression was in cells at the dorsal midline, in or near the roof plate (Figures 4C, 4E, 4F, 4H, and 4I). In several embryos, the morphology of the neural tube in regions of ectopic floor plate markers expression was abnormal with marked constrictions or folds in the neural tube (data not shown).

#### Floor Plate Differentiation Induced in Vitro by *vhh-1*

To test more directly the ability to *vhh-1* to induce ventral neural cell types, applicants used established in vitro assays of rat floor plate (Placzek et al., 1993) and chick motor neuron (Yamada et al., 1993) differentiation.

To detect floor plate differentiation, applicants monitored the induction of the floor plate antigens FP3 and FP4 (Figures 5A and 5B) in rat neural plate explants cultured in vitro. Notochord and floor plate induce the expression of FP3 and FP4 when grown in contact with E9-E10 rat neural plate tissue (Figures 5C and 5D) (Placzek

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et al., 1993). Expression vectors containing full-length *vhh-1* cDNA in sense or antisense orientations were transiently transfected into COS cells. About 25% of COS cells expressed *vhh-1* RAN (data not shown).

5

Of neural plate explants grown in contact with COS cells expressing sense *vhh-1* cDNA, 70% expressed FP3 and 47% expressed FP4 (Figures 5E-5H; Table 1). As with floor plate induction by the notochord, not all explants that expressed FP3 also expressed FP4. This may reflect the later onset of FP4 expression in vivo (Placzek et al., 1993). The domain of FP3 and FP4 expression within neural plate explants was similar in size to that induced by the notochord, and labeled cells were located close to the junction of the COS cells aggregate and neural plate explant. Induction of floor plate differentiation by *vhh-1* may thus be local and possibly contact-dependent process. Consistent with this, medium harvested from *vhh-1* transfected COS cells did not induce FP3 or FP4 when added to neural plate explant grown alone (data not shown). It remains to be determined, however, whether *vhh-1* activity can diffuse into the medium. Neural plate explants grown in contact with cells transfected with antisense *vhh-1* cDNA did not express FP3 or FP4 (Figures 5J and 5K; Table 1).

25

The simplest explanation of these results is that *vhh-1* protein is secreted from COS cells and interacts with neural plate cells to trigger, directly, floor plate differentiation. Nevertheless, it remains possible that expression of *vhh-1* in COS cells induces the synthesis of a distinct factor that mediates floor plate induction. In addition, these results do not resolve whether the *vhh-1* protein is sufficient to induce floor plate differentiation since COS cells could provide an

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accessory factor that acts in concert with the vhh-1 protein.

Motor Neuron Differentiation Induced In Vitro by vhh-1

5

In vitro studies have provided evidence that signals from the notochord can induce the differentiation of motor neurons as well as floor plate cells (Yamada et al., 1993). The expression of vhh-1 in the notochord therefore raises the questions of whether motor neurons  
10 can also be induced by vhh-1.

To determine whether vhh-1 can also induce motor neurons, applicants used chick neural plate explants in which  
15 motor neuron differentiation has been characterized (Table 1; Yamada et al., 1993). Motor neurons can be identified by the coexpression of two markers, the LIM homeodomain protein islet -1 (Thor et al., 1991; Ericson et al., 1992) (Figure 6A) and the immunoglobulin-like  
20 protein SC1 (Tanaka and Obata, 1984) (Figure 6D). Intermediate neural plate explants (Yamada et al., 1993) were grown for 44 hrs on a monolayer of COS cells transfected with sense or antisense vhh-1 expression plasmids. Neural plate explants grown on COS cells  
25 expressing the sense cDNA contained an average of 83 Islet-1' cells (Figures 6B and 6C; Table 1), whereas explants grown on COS cells transfected with antisense vhh-1 cDNA expressed at most one islet-1' (Figure 6G, Table 1, motor neuron induction). Immunofluorescence  
30 labelling and confocal imaging revealed that most islet-1' cells expressed SC1 on their surface (Figures 6E and 6F) (n = 27 explants), confirming their identity as motor neurons. Medium conditioned by COS cells transfected with sense vhh-1 cDNA did not induce islet-1' calls in  
35 intermediate neural plate explants (date not shown).

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Since ambiguous markers of floor plate differentiation in chick neural plate explants are not available, applicants could not assay whether floor plate differentiation also occurs in chick neural plate explants in response to vhh-1.

Taken together, these in vitro assays provide evidence that COS cells expressing vhh-1 can induce both floor plate cells and motor neurons, although it is unclear whether motor neuron induction is a direct response to vhh-1.

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Table 1. Induction of Floor Plate and Motor Neuron Differentiation in Neural Plate Explants in Vitro

Inducer	Floor Plate Induction <sup>a</sup>			Motor Neuron Induction <sup>b</sup>	
	Percentage FP3 <sup>+</sup> Explants	Percentage FP4 <sup>+</sup> Explants	n (Explants)	Number of Islet-1 <sup>+</sup> Cells	n (Explants)
Notochord <sup>c</sup>	85	63	65, 30	210 ± 12	22
vhh-1 COS cells	70	47	47	83 ± 8	24
Antisense vhh-1 COS cells	0	0	16	0 - 1	20
Floor plate-conditioned medium				60 ± 4	20
Posterior limb mesenchyme	73	45	22		
Anterior limb mesenchyme	0	0	22		

<sup>a</sup>Numbers derive from three to six separate experiments.

<sup>b</sup>Values given are means ± SEM from 1 of 6 similar experiments; caudal stage 10 notochord was used. Floor plate-conditioned medium was prepared as described by Yamada et al. (1993).

<sup>c</sup>Data for floor plate induction from Placzek et al. (1993).

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Floor Plate Differentiation Is Induced In Vitro by  
Posterior Limb Bud Calls

5 The node, notochord, and floor plate can induce floor  
plate differentiation (Placzek et al., 191, 1993) and can  
also mimic the ability of the ZPA to evoke digit  
10 duplications in the developing chick limb bud (Hornbruch  
and Wolpert, 1986; Wagner et al., 1990, Stoker and  
Carison, 1990; Hogan et al., 1992). The expression of  
vhh-1 in the ZPA region (see Figure 3; Figure 7A) raises  
the questions of whether the ZPA can mimic the ability of  
midline cells to induce floor plate differentiation. To  
test this, applicants assayed the ability of the ZPA to  
15 induce floor plate differentiation in rat neural plate  
explants in vitro. The ZPA region of the posterior limb  
mesenchyme (Honig and Summerbell, 1985) was isolated  
together with the adjacent apical ectoderm to provide  
factors that maintain ZPA activity in vitro (Anderson, et  
al., 1993; Vogel and Tickle, 1993; Niswander et al.,  
20 1993). Of neural plate explants grown in contact with  
posterior limb mesenchyme and ectoderm, 73% expressed FP3  
and 45% displayed FP4 (Table 1, floor plate induction;  
Figures 7B and 7C). In contrast, neural plate explants  
grown in contact with anterior limb mesenchyme and  
25 ectoderm did not express FP3 or FP4 (Figures 7D and 7E;  
Table 1, floor plate induction). Neural plate explants  
grown in contact with posterior limb ectoderm in the  
absence of mesenchyme did not induce FP3 or FP4 (data not  
shown). These results support the idea that vhh-1  
30 expression confers cells with floor plate inducing  
properties.

EXPERIMENTAL DISCUSSION

35 The differentiation of ventral cell types within the

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neural tube is controlled by signals that derive from the notochord. Applicants have identified a vertebrate homolog of the *Drosophila hh* gene, *vhh-1*, that is expressed in midline mesodermal and neural cells: the node, the notochord, and the floor plate. Widespread expression of the *vhh-1* gene in frog embryos leads to ectopic floor plate differentiation, and COS cells expressing *vhh-1* can induce floor plate and motor neuron differentiation in neural plate explants in vitro. Our results suggest that expression of *vhh-1* by the notochord participates in the induction of floor plate and motor neuron differentiation in overlying neural plate cells.

Involvement of *vhh-1* in Floor Plate and Motor Neuron Differentiation

In vitro studies have provided evidence for two distinct activities of the notochord, a contact mediated floor plate inducing activity and a diffusible motor neuron inducing activity (Placzek et al., 1990a, 1990b, 1993; Yamada et al., 1993). Both activities are also acquired by the floor plate after its induction by the notochord. Our results provide evidence that floor plate induction occurs as a direct response to *vhh-1*. Moreover, as with the notochord derived signal, floor plate induction by *vhh-1* appears to be a local event and may be contact mediated.

Although *vhh-1* can induce motor neurons as well as floor plate cells, our results do not resolve whether this induction is direct and thus whether *vhh-1* could represent the diffusible motor neuron inducing activity present in notochord- and floor plate-conditioned medium. Since *vhh-1* can induce floor plate differentiation, the induced floor plate could, in turn, secrete a motor

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neuron-inducing factor distinct from *vhh-1*. It is also unclear whether *vhh-1* is present in medium conditioned by cells that secrete *vhh-1*. In *Drosophila*, *hh* is known to act nonautonomously (Mohler, 1988), and analysis of *hh* (or a downstream mediator of *hh* function) can act over a distance of a few cell diameters (Ingham, 1993; Heberlein et al., 1993; Ma et al., 1993; Heemskerk and Dinardo, 1994; Basier and Struhl, 1994). Consistent with this, *hh* protein has been detected beyond the domain of *hh* mRNA expression (Taylor et al., 1993).

The early expression of *vhh-1* by the notochord is synchronous with its floor plate and motor neuron inducing activities. However, the persistent expression of *vhh-1* by the notochord at later stages of embryonic development contrasts with in vitro studies showing that the notochord rapidly loses its ability to induce floor plate in vitro (Placzek et al., 1990a, 1990b, 1993). This difference could reflect the onset of expression of notochord factors that inhibit the action of *vhh-1* or the loss of expression of a required cofactor. In rat, *vhh-1* expression by floor plate cells can first be detected after neural tube closure, consistent with the time at which floor plate cells acquire floor plate and motor neuron inducing activity (Placzek et al., 1993; Yamada et al., 1993). By this time it appears that cells in the neural plate have been exposed to signals that initiate more neuron differentiation (Yamada et al., 1993). It is unlikely, therefore, that *vhh-1* expression by the floor plate is involved in the initiation of motor neuron differentiation. Nevertheless, it is possible that later-born motor neurons (Hollyday and Hamburger, 1977) depend on floor plate-derived *vhh-1* for their differentiation. A second function of *vhh-1* in the floor plate may be to participate in the recruitment of



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additional cells to the floor plate as the neural tube grows (Placzek et al., 1993).

#### Pathway of Floor Plate Differentiation

5 The ability of *vhh-1* to induce ectopic HNF-3 $\beta$  in the neural tube may be relevant to the steps involved in the normal development of the floor plate. *Pintallavis* and HNF-3 $\beta$  are expressed in the node, notochord, and floor plate (Ruiz i Altaba and Jessell, 1992; Monaghan et al., 10 1993; Sasaki and Hogan, 1993; Ruiz i Altaba et al., 1993b). The expression of both genes by the floor plate is dependent on inductive signals from the notochord (Ruiz i Altaba et al., 1992; A.R.A., MP., J.D., AND 15 T.M.J., unpublished data), and expression occurs before other floor plate properties.

Widespread expression of *Pintallavis* and HNF-3 $\beta$  induces the expression of floor plate markers in the dorsal 20 neural tube (Ruiz i Altaba et al., 1993a; A.R.A. et al., unpublished data; Sasaki and Hogan, 1994), suggesting that HNF-3 $\beta$  and *Pintallavis* are involved in the specification of floor plate fate in cells at the midline of the neural plate. The induction of HNF-3 $\beta$  by *vhh-1*, 25 therefore, appears to mimic the ability of the notochord to trigger a program of floor plate differentiation that includes the transcription of genes such as *vhh-1* itself and F-spondin.

#### Requirements for Floor Plate Differentiation

Widespread expression of rat *vhh-1* in frog embryos induces ectopic floor plate differentiation in vivo. The chick and zebrafish *shh* genes have also been shown to 35 induce floor plate markers, although only in midbrain

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regions (Echelard et al., 1993; Krauss et al., 1993). Our in vivo studies show clearly that atopic expression of floor plate markers can also be obtained at hindbrain and spinal cord levels, although not in the forebrain. The absence of ectopic floor plate markers in the forebrain is consistent with in vitro studies showing that notochord cannot induce floor plate differentiation in anterior regions of the neural plate (Placzek et al., 1993).

Although widespread expression of *vhh-1* in frog embryos induces ectopic floor plate differentiation in vivo. The chick and zebrafish *shh* genes have also been shown to induce floor plate markers, although only in midbrain regions (Echelard et al., 1993; Krauss et al., 1993). Our in vivo studies show clearly that atopic expression of floor plate markers can also be obtained at hindbrain and spinal cord levels, although not in the forebrain. The absence of ectopic floor plate markers in the forebrain is consistent with in vitro studies showing that notochord cannot induce floor plate differentiation in anterior regions of the neural plate (Placzek et al., 1993).

Although widespread expression of *vhh-1* induces ectopic floor plate differentiation at all levels of the neuraxis caudal to the forebrain, applicants observed that ectopic floor plate markers were found primarily in the dorsal region of the neural tube. Notochord grafts can, however, induce floor plate differentiation at all dorsoventral positions within the neural tube (van Straaten et al., 1988; Yamada et al., 1991). Thus signals from the notochord may, in vivo, induce floor plate differentiation in regions of the neural tube that do not respond to *vhh-1* alone. The observed differences

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in neural tube responses to *vhh-1* and to the notochord could result from quantitative differences in *vhh-1* levels provided by the notochord and by the *vhh-1* expression plasmid. Alternatively, the notochord may provide additional signaling molecules, one function of which could be to regulate the expression of transcription factors that cooperate with *Pintallavis* and *HNF-3 $\beta$*  in the determination of floor plate fate.

10 *Vhh-1* Expression and the Reciprocity of Neural Tube  
and Limb Polarizing Activities

The expression of *vhh-1* in the node, notochord, floor plate and posterior limb mesenchyme provides a possible molecular basis for the shared signaling properties of these cell groups (Jessell and Dodd, 1992; Ruiz l Altaba and Jessell, 1993). Grafts of Hensen's node, the notochord, or floor plate into the anterior region of the developing chick limb bud evoke digit duplications that mimic those of the ZPA (Hornbruch and Wolpert, 1986; Wagner et al., 1990; Stoker and Carlson, 1990; Hogan et al., 1992). The present results show that the ZPA can induce floor plate differentiation. Moreover, the common signaling properties of the node, notochord, floor plate, and ZPA appear to correlate more closely with the pattern of *vhh-1* expression than with retinoid activity (Thaller and Eichele, 1987; Rossant et al., 1991; Wagner et al., 1992). Additional support for the idea that the limb and neural patterning have a common basis is provided by recent studies showing that chick *shh* can mimic ZPA activity when expressed in anterior regions of the limb bud (Riddle et al., 1993). Expression of the *vhh-1* gene in the node, notochord, and floor plate is likely, therefore, to underlie the ability of these midline cell groups to mimic the activity of the ZPA in evoking digit

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duplications. Reciprocally, the expression of *vhh-1* may underlie the ability of the ZPA to induce floor plate differentiation.

5     Hh-Related TGF $\beta$  and Wnt Proteins as Secreted  
      Regulators of Cell Pattern

10     In *Drosophila*, *dpp*, *wg*, and *hh* regulate cell fate and pattern in embryonic and larval development. In  
vertebrates, members of the TGF $\beta$  and *wnt* gene families regulate cell differentiation during neural development. The *wnt-1* gene is required for midbrain and anterior hindbrain development (McMahon and Bradley, 1990; Thomas and Capecchi, 1990), and *dorsalin-1*, a member of the TGF $\beta$   
15     family, promotes the differentiation of dorsal cell types in neural plate explants in vitro (Blaser et al., 1993). Our results suggest that *vhh-1* also contributes to neural patterning in vertebrates, acting to induce distinct cell types in the ventral region of the neural tube. Thus,  
20     *dorsalin-1* dorsally and *vhh-1* ventrally may provide polarizing signals with opposing actions that specify cell fates along the dorsoventral axis of the neural tube.

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Second Series of Experiments

The vertebrate hedgehog-related gene, *vhh-1/sonic hedgehog*, is expressed in ventral domains along the entire rostrocaudal length of the neural tube, including the forebrain. Applicants show here that *vhh-1/shh* induces the differentiation of ventral neuronal cell types in explants derived from prospective forebrain regions of the neural plate. Neurons induced in explants derived from both diencephalic and telencephalic levels of the neural plate express the LIM homeodomain protein Islet-1, but these neurons possess distinct identities that match those of the ventral neurons normally generated in these two subdivisions of the forebrain. These results, together with previous studies of neuronal differentiation at caudal levels of the neural tube suggest that a single inducing molecule, *vhh-1/shh*, mediates the induction of distinct ventral neuronal cell types along the entire rostrocaudal extent of the embryonic central nervous system.

In vertebrate embryos, the patterning of the nervous system is initiated by inductive signals that act over short distances to direct the fate of neural progenitor cells. The complex pattern of cell types generated within the neural tube is thought to involve the action of signals that impose regional character on cells at different rostrocaudal positions within the neural plate (Doniach et al., 1992; Ruiz i Altaba, 1992; Papalopulu, 1994) and that define the identity of cells along the dorsoventral axis of the neural tube (Jessell and Dodd, 1992; Basler et al. 1993; Smith, 1993). Thus, the fate of neural progenitor cells depends on their position along the rostrocaudal and dorsoventral axes of the neural tube.

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The mechanisms that control the differentiation of cell types along the dorsoventral axis of the neural tube have been examined in most detail at caudal levels of the neuraxis. In the spinal cord, the differentiation of ventral cell types is initiated by signals transmitted from axial mesodermal cells of the notochord to overlying neural plate cells, inducing the differentiation of floor plate cells at the ventral midline and motor neurons more laterally within the neural tube (van Straaten et al., 1988; Placzek et al., 1990; 1991; Yamada et al., 1991, 1993; Goulding et al., 1993). At later stages, similar or identical signalling properties are acquired by floor plate cells (Hatta et al., 1991; Yamada et al. 1991; Placzek et al., 1993). The specific identity of the ventral neuronal cell types that are generated in response to notochord- and floor plate-derived signals, however, appears to be defined by the position of origin of neuronal progenitor cells along the rostrocaudal axis. For example, serotonergic neurons are induced by midline-derived signals at the level of the rostral rhombencephalon (Yamada et al., 1991) whereas dopaminergic neurons are induced at the level of the mesencephalon (Hynes et al., 1995).

At caudal levels of the neuraxis, a vertebrate homolog of the secreted glycoprotein encoded by the *Drosophila* gene *hedgehog* (Nusslein-Volhard and Wieschaus 1980; Lee et al., 1992), *vhh-1/sonic hedgehog (shh)*, has been implicated in the induction of ventral cell types. *vhh-1/shh* is expressed by the notochord and floor plate at the time that these two cell groups exhibit their inductive activities (Riddle et al., 1993; Krauss et al., 1993; Echelard et al., 1993; Chang et al., 1994; Roelink et al., 1994). Furthermore, exposure of neural plate explants to *vhh-1/shh* leads to the differentiation of

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motor neurons in addition to floor plate cells (Roelink et al., 1994), suggesting that *vhh-1/shh* participates in the induction of ventral neurons at caudal levels of the neuraxis.

5

At most levels of the embryonic forebrain, the notochord and floor plate are absent (Kingsbury, 1930; Puellès and Rubenstein, 1993) and neither the identity nor the source of inductive signals that trigger the differentiation of ventral neurons have been established. Studies of the zebrafish mutant *cyclops* (Hatta et al., 1991) have provided evidence that cells at the ventral midline of the embryonic diencephalon have a role in the patterning of the diencephalon (Hatta et al., 1994; Macdonald et al., 1994). *vhh-1/shh* is expressed by cells at the ventral midline of the embryonic forebrain (Echelard et al., 1993; Krauss et al., 1993; Chang et al., 1994; Roelink et al., 1994), raising the possibility that this gene participates in the specifications of neuronal identity within the forebrain as well as at more caudal levels in the neuraxis.

To address this issue, applicants first defined transcription factors and other molecular markers that permit the identification of ventral neuronal cell types generated in diencephalic and telencephalic subdivisions of the forebrain. Applicants then used these markers to assess the ability of *vhh-1/shh* to induce the differentiation of distinct ventral neuronal classes in explants derived from levels of the neural plate fated to give rise to the forebrain. Applicants' results show that *vhh-1/shh* induces ventral neuronal cell types normally found in the forebrain in addition to inducing motor neurons at more caudal levels of the neural tube. These findings suggest that a single inducing molecule,

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vhh-1/shh, is responsible for inducing ventral neuronal cell types along the entire rostrocaudal extent of the neuraxis. They also indicate that the repertoire of ventral neuronal cell types that can be induced by vhh-1/shh is defined by an earlier restriction in the rostrocaudal character of cells within the neural plate.

#### EXPERIMENTAL RESULTS

##### vhh-1/shh and Islet-1 Occupy Adjacent Ventral Domains in the Embryonic CNS

To begin to examine the involvement of vhh-1/shh in the patterning of the embryonic forebrain, it was necessary to identify early markers of ventral forebrain neurons. At caudal levels of the neuraxis, motor neurons constitute one prominent class of ventral neuron whose differentiation depends on inductive signals provided by the notochord and floor plate (Yamada et al., 1991, 1993). The earliest marker of differentiating motor neurons is Islet-1 (Karlsson et al., 1990), a LIM homeodomain protein that is expressed as motor neuron progenitors leave the cell cycle (Ericson et al., 1992; Korzh et al., 1993; Inoue et al., 1993; Tsuchida et al., 1994). Although motor neurons are absent from the forebrain, Islet-1 is expressed by ventral neurons in the adult forebrain (Thor et al., 1991). This observation prompted applicants to examine whether the embryonic expression of Islet-1 provides an early marker of the differentiation of ventral neuronal cell types at forebrain as well as at more caudal levels of the neuraxis.

Applicants therefore examined the pattern of expression of Islet-1 in the embryonic chick nervous system and

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compared it to that of *vhh-1/shh*. At Hamburger-Hamilton (HH) stage 18, Islet-1<sup>+</sup> cells were found in discrete domains along the rostrocaudal axis of the neural tube. Each Islet-1<sup>+</sup> cell group abutted the domain of expression of *vhh-1/shh* (Fig. 8, see Fig. 9Ai for a summary). In the spinal cord, rhombencephalon and mesencephalon, *vhh-1/shh* was expressed by floor plate cells at the ventral midline (Fig. 8B, F, G and data not shown) and Islet-1 was expressed by cells located lateral to the floor plate (Fig. 8B, F, G and data not shown). In the mid-diencephalon at the level of the infundibulum, *vhh-1/shh* was not expressed at the ventral midline but was located more laterally (Fig. 8A, D). Islet-1<sup>+</sup> cells were also excluded from the ventral midline but were located immediately lateral to the zone of *vhh-1/shh* expression (Fig. 8D). In the rostral diencephalon, *vhh-1/shh* was expressed at the ventral midline of the neural tube and was restricted to the ventricular zone (Fig. 8E, H, I). Within this region, Islet-1<sup>+</sup> cells were also located at the midline, immediately adjacent to the domain of expression of *vhh-1/shh* (Fig. 8I). In the telencephalon, the zone of *vhh-1/shh* expression also spanned the ventral midline of the neural tube (Fig. 8J, K). Islet-1<sup>+</sup> cells were also restricted ventrally and were intermingled with cells expressing *vhh-1/shh* (Fig. 8K). These results indicate that Islet-1 expression defines ventral cell types at forebrain as well as at more caudal levels of the neural tube.

At all levels of the neuraxis, with the exception of the telencephalon, the expression of *vhh-1/shh* preceded the differential of Islet-1<sup>+</sup> cells. Expression of *vhh-1/shh* was detected in cells at the midline of the neural plate at prospective mesencephalic levels at HH stage 6 (Fig. 10A; and not shown). Between HH stages 6 and 10, midline

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expression of *vhh-1/shh* extended rostrally into the prospective diencephalon and caudally into the rhombencephalon and spinal cord (data not shown). The onset of Islet-1 expression at spinal cord, rhombencephalic, mesencephalic and diencephalic levels occurred between HH stages 13 and 15 (Fig. 8E; Ericson et al., 1992; Tsuchida et al., 1994; and data not shown), 18-24 hours after the onset of *vhh-1/shh* expression at similar axial levels. In the ventral telencephalon, however, expression of *vhh-1/shh* was not detected until late HH stage 17, about 30 hours after the gene was first expressed in ventral midline cells of the rostral diencephalon (data not shown) and coincident with the onset of Islet-1 expression.

15

Cells that Express Islet-1 at Different Axial Levels are Neurons with Distinct Identities

To determine whether the ventral Islet-1<sup>+</sup> cells detected at different rostrocaudal levels of the neuraxis were neurons, applicants performed double-label immunocytochemistry with antibodies directed against Islet-1 and the neuron-specific markers  $\beta$ -tubulin and cyn-1. At all axial levels, Islet-1<sup>+</sup> cells expressed  $\beta$ -tubulin and/or cyn-1, confirming their identity as neurons (data not shown). Although all Islet-1<sup>+</sup> cells were neurons, however, their identities at different rostrocaudal positions were distinct.

30

SC1 Expression Defines Islet-1<sup>+</sup> Neurons as Motor Neurons:

In the rhombencephalon and mesencephalon, the location of Islet-1<sup>+</sup> neurons coincided with the positions of somatic, visceral and brachial motor nuclei. At these levels, Islet-1<sup>+</sup> neurons expressed the immunoglobulin-like surface protein SC1 (Fig. 9Aii, B and data not shown), in common

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with spinal motor neurons (Yamada et al., 1991; Ericson et al.). The rostral-most group of motor neurons is generated in the mesencephalon (see Simon et al., 1994), thus Islet-1<sup>+</sup> neurons found in the embryonic diencephalon and telencephalon are unlikely to give rise to motor neurons. Consistent with this, neither diencephalic nor telencephalic Islet-1<sup>+</sup> neurons expressed SC1 (Fig. 9C and data not shown, see also Table 3).

10 Nkx 2.1 Expression Defines Ventral Forebrain Cells: To identify a marker with which to distinguish cells in diencephalic and telencephalic regions from those found more caudally, applicants examined the pattern of expression of the homeodomain-containing protein Nkx 2.1.

15 In mouse embryos, Nkx 2.1 mRNA is expressed at prospective diencephalic and telencephalic levels of the neural tube in a ventral domain that overlaps with that of *vhh-1/shh*, but the gene is not expressed at rhombencephalic or spinal cord levels (Lazzaro et al.,

20 1991; Price et al., 1992; Rubenstein et al., 1994). In chick embryos examined at HH stages 14-18, antibodies directed against Nkx 2.1 labeled cells in a broad ventral domain of the mid and rostral diencephalon and telencephalon (Fig. 9Aiii, D and data not shown) Nkx 2.1<sup>+</sup>

25 cells were not detected in the rhombencephalon or spinal cord (Fig. 9Aiii and data not shown). The onset of expression of Nkx 2.1 in the diencephalon occurred at HH stage 9 and in the telencephalon at HH stage 13/14 (data not shown). The expression of Nkx 2.1 in the ventral

30 forebrain was transient, and by HH stages 19-20 the number of Nkx 2.1<sup>+</sup> cells had decreased markedly (data not shown). Because of this, it was difficult to determine accurately the extent of overlap between cells that expressed Nkx 2.1 and Islet-1. However, when examined at

35 HH stage 18, about 10% of Nkx 2.1<sup>+</sup> cells coexpressed

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Islet-1 (data not shown). Thus, the expression of Nkx 2.1 serves primarily as a marker of ventral forebrain cells but coexpression of Nkx 2.1 and Islet-1 can be used to distinguish Islet-1<sup>+</sup> neurons generated in the diencephalon and telencephalon from those found at more caudal levels.

Lim-1 Expression Distinguishes Diencephalic and Telencephalic Cells: To identify a marker with which to distinguish Islet-1<sup>+</sup> neurons in the diencephalon from those in the telencephalon, applicants examined the expression of the LIM homeodomain protein Lim-1 (Taira et al., 1992). In the embryonic mouse forebrain, *Lim-1* mRNA is restricted almost exclusively to the diencephalon (Barnes et al., 1994, Fujii et al., 1994). In chick embryos examined from HH stages 14-18, antibodies directed against Lim-1 (Tsuchida et al., 1994) detected cells in the diencephalon in a pattern similar to that described for Lim-1 mRNA in mouse (see Fig. 9Aii). At these stages Lim-1<sup>+</sup> cells were not detected in the telencephalon (Fig. 9A, and data not shown). Applicants next examined the relationship between Lim-1<sup>+</sup> cells and Islet-1<sup>+</sup> neurons in the diencephalon at HH stages 14-18. In the mid-diencephalon, but not at other levels of the diencephalon, Lim-1 was expressed by neuroepithelial cells (Fig. 9Aii, F). At this axial level, Lim-1<sup>+</sup> neurons were also present, moreover the majority of Islet-1<sup>+</sup> neurons expressed Lim-1 (Fig. 9E, F). In the rostral diencephalon, Lim-1 was expressed in the same population of ventral midline neurons that expressed Islet-1 (Fig. 9G-I). In the intervening region of the diencephalon, Lim-1<sup>+</sup> neurons were also present in a population distinct from, but intermingled with, Islet-1<sup>+</sup> neurons (Fig. 9Aii). In the telencephalon, Islet-1<sup>+</sup> neurons did not express Lim-1 (Fig. 9J). Thus, Lim-1 expression distinguishes

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diencephalic from telencephalic cells. Moreover, although Lim-1 is not a marker of all diencephalic Islet-1<sup>+</sup> neurons, its coexpression with Islet-1 indicates the diencephalic origin of Islet-1<sup>+</sup> forebrain neurons.

5

vhh-1/shh Induces Islet-1<sup>+</sup> Neurons in Prospective Forebrain Regions of the Neural Plate

10 In order to isolate explants from regions of the neural plate that give rise to defined rostrocaudal domains of the neural tube, applicants constructed a coarse fate map of the neural plate of HH stage 6 chick embryos (see Experimental Procedures). This map was then used as a guide to isolate explants from lateral regions of the  
15 neural plate at three different levels of the neuraxis: i) a level ([T] in Fig. 10A) fated to give rise to the telencephalon; ii) a level ([D] in Fig. 10A) fated to give rise to the diencephalon, and iii) a level ([R] in Fig. 10A) fated to give rise to the rhombencephalon.  
20 Applicants then used the markers described above to examine whether vhh-1/shh can induce the differentiation of ventral neurons in explants derived from prospective forebrain levels of the neural plate as well as from more caudal levels.

25

Applicants examined first the expression of Islet-1 by cells in neural plate explants obtained from telencephalic, diencephalic and rhombencephalic levels grown in the absence of vhh-1/shh. Neural plate explants  
30 were grown for 60-66 hours in vitro, in the presence of COS cells transfected with antisense vhh-1 cDNA. Under these conditions, cells in explants derived from all three axial levels expressed the neuronal marker  $\beta$ -tubulin but Islet-1<sup>+</sup> cells were not detected (Fig. 10, B, C, F, G, J, K). In contrast, numerous Islet-1<sup>+</sup> cells were  
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induced in explants derived from each of the three axial levels of the neural plate when they were grown on COS cells transfected with sense *vhh-1/shh* cDNA (Fig. 10D, E, H, I, L, M, Table 2). The proportion of Islet-1<sup>+</sup> neurons in induced explants derived from the three axial levels differed markedly. In telencephalic level explants, 96% of cells exposed to *vhh-1/shh* expressed Islet-1 (Table 2) whereas only 35% of cells in diencephalic level explants and 39% of cells in rhombencephalic level explants expressed Islet-1 (Table 2).

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Table 2Induction of Islet-1<sup>+</sup> cells by vhh-1/shh in Neural Plate Explants

	<u>Region of Neural Plate</u>	<u>Transfection construct</u>	(%) <u>Islet-1<sup>+</sup> explants</u>
5			
10			
		Antisense vhh-1/shh	0 (49)
	Rhombencephalic:	Sense vhh-1/shh	57 (45)
15			
		Antisense vhh-1/shh	0 (28)
	Diencephalic:	Sense vhh-1/shh	57 (30)
20			
		Antisense vhh-1/shh	0 (46)
	Telencephalic:	Sense vhh-1/shh	78 (42)

Table 2 - Cont'd

	<u>Region of Neural Plate</u>	<u>Transfection construct</u>	(%) <u>Islet-1<sup>+</sup> neurons/ explant</u>	(%) <u>Islet-1<sup>+</sup> neurons that express Lim-1</u>
25				
30				
		Antisense vhh-1/shh	0	-
35	Rhombencephalic:	Sense vhh-1/shh	39 (11)	0 (16)
		Antisense vhh-1/shh	0	-
40	Diencephalic:	Sense vhh-1/shh	35 (9)	22 (11)
		Antisense vhh-1/shh	0	0
	Telencephalic:	Sense vhh-1/shh	96 (7)	0 (15)
45				

Neural plate explants isolated from telencephalic, diencephalic and rhombencephalic levels of HH stage 6 chick embryos were cultivated for 60-66 hours in contact with COS cells transfected with a vhh-1 expression construct in sense or antisense orientation and the proportion of explants that express Islet-1 was determined by whole mount

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5 immunohistochemistry. The percentage of Islet-1<sup>+</sup> and Lim-1<sup>+</sup> cells in vhh-1/shh-induced explants was determined by sectioning explants and counting the number of labeled cells in individual sections. The total number of cells in explants was determined using DAPI nucleic staining. The number of explants analyzed is indicated in brackets.

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Islet-1<sup>+</sup> Neurons Induced by vhh-1/shh Have Distinct Axial Identities

- 5 To assess the rostrocaudal character of cells in neural plate explants derived from different axial levels and in particular to define the identity of induced Islet-1<sup>+</sup> neurons, applicants examined the expression of SC1, Nkx 2.1 and Lim-1.
- 10 SC1 Expression: Neural plate explants did not express SC1 when grown on COS cells transfected with antisense vhh-1/shh cDNA (Table 3). In rhombencephalic level explants that had been exposed to vhh-1/shh, Islet-1<sup>+</sup> neurons expressed SC1 (Fig. 11, A, B), indicating that
- 15 these cells are motor neurons. However, Islet-1<sup>+</sup> neurons accounted for only about 50% of the SC1<sup>+</sup> cells induced by vhh-1/shh in rhombencephalic explants. The remaining, Islet-1<sup>+</sup>/SC1<sup>+</sup> cells (Fig. 11C, D) expressed the FPI marker (data not shown) indicating that they are floor plate
- 20 cells (Yamada et al., 1991). In diencephalic and telencephalic level explants, the Islet-1<sup>+</sup> neurons induced by exposure to vhh-1/shh did not coexpress SC1 (Fig. 11, E, F, J, I) providing evidence that they are not motor neurons. Floor plate cells, defined by expression of
- 25 FPI, were not detected in diencephalic or telencephalic level explants exposed to vhh-1/shh (data not shown).

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Table 3Marker Expression in Explants Derived from Different Axial Levels of the Neural Plate

	<u>Region of Neural Plate</u>	<u>Transfection Construct</u>	<u>Marker Expression</u>		
			<u>Islet-1</u>	<u>SC1</u>	<u>Nx2.1</u>
5					
10		-----			
	Rhombencephalic:	Antisense vhh-1/shh	-	-	-
		Sense vhh-1/shh	++	++	-
15		-----			
	Diencephalic:	Antisense vhh-1/shh	-	-	-
		Sense vhh-/shh	++	-	+
20		-----			
	Telencephalic:	Antisense vhh-1/shh	-	-	-
		Sense vhh-1/shh	+++	-	+
		-----			

Table 3 - Cont'd

	<u>Region of Neural Plate</u>	<u>Transfection Construct</u>	<u>Lim-1</u>
25			
30		-----	
	Rhombencephalic:	Antisense vhh-1/shh	++
		Sense vhh-1/shh	+
35		-----	
	Diencephalic:	Antisense vhh-1/shh	++
		Sense vhh-1/shh	++
40		-----	
	Telencephalic:	Antisense vhh-1/shh	-
		Sense vhh-1/shh	-
		-----	

45 Analysis of neural plate explants grown for 60-66 hours in contact with COS cells transfected with either sense or antisense vhh-1 expression constructs. (-) sign indicates that fewer than 0.5%, (+) 5-35%, (++) 35-80%, (+++) >90% of cells expressed the marker, n.d. = not determined. Results were obtained from over 30 explants in each case.



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Nkx 2.1 Expression: Neural plate explants did not express Nkx 2.1 when grown on COS cells transfected with antisense *vhh-1/shh* cDNA (Table 3). Moreover, Nkx 2.1<sup>+</sup> cells were not detected in rhombencephalic level explants exposed to *vhh-1/shh* (Fig. 12A) whereas induced diencephalic and telencephalic level explants contained Nkx 2.1<sup>+</sup> cells (Fig. 12B, C), and after 60-66 hours in vitro 5-10% of cells coexpressed Islet-1 (data not shown).

Lim-1 Expression: Lim-1<sup>+</sup> cells were detected in rhombencephalic (Table 3) and diencephalic (Fig. 12D) but not telencephalic (Fig. 12G) level explants grown on COS cells transfected with antisense *vhh-1* cDNA. In diencephalic level explants exposed to *vhh-1/shh*, 22% of Islet-1<sup>+</sup> neurons expressed Lim-1 (Fig. 12, E, F, Table 2) and thus correspond phenotypically, to neurons characteristic of the diencephalon (Fig. 9Aii). In contrast, in both rhombencephalic and telencephalic level explants, the Islet-1<sup>+</sup> neurons induced by *vhh-1/shh* did not express Lim-1 (Fig. 12, H, I, Table 2).

Taken together, these in vitro experiments show that *vhh-1/shh* induces ventral neuronal cell types in prospective forebrain regions of the neural plate and that these neurons express marker combinations appropriate for distinct classes of ventral neurons that are generated ventrally in both the diencephalon and telencephalon.

Floor Plate and Midline Rostral Diencephalic Cells Mimic the Inductive Actions of *vhh-1/shh*

The results described above leave open the possibility that the inducing activity of *vhh-1/shh* expressed in COS cells differs from the activities of neural cell groups

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implicated in the induction of ventral neurons in vivo. Applicants therefore determined whether the response of neural plate explants to *vhh-1/shh* was mimicked by potentially relevant neural sources of *vhh-1/shh*.

5 Applicants assayed the activity of chick floor plate as a source of *vhh-1/shh* implicated in the induction of ventral cell types at spinal cord, rhombencephalic and mesencephalic levels (Fig. 8). Floor plate tissue induced Islet-1<sup>+</sup> neurons in rhombencephalic level neural  
10 plate explants (Fig. 13A) and these neurons coexpressed SC1 (data not shown). *Nkx 2.1*<sup>+</sup> cells were not induced in rhombencephalic level explants by floor plate tissue (Fig. 13B). Thus, the inductive activity of floor plate was similar to that of *vhh-1/shh* expressed in COS cells.

15 Applicants also assayed the activity of cells at the ventral midline of the rostral diencephalon that express *vhh-1/shh* (Fig. 8) as a neural source of *vhh-1/shh* that might be involved in the patterning of the diencephalon  
20 (Hatta et al., 1994) and ventral telencephalon (see Experimental Discussion). Since the midline of the rostral diencephalon itself expresses Islet-1<sup>+</sup> neurons, midline diencephalic inducing tissue was derived from E11 mouse embryos and species-specific antibodies directed  
25 against the intermediate filament protein nestin (Dahlstrand et al., 1992) were used to define the murine inducing tissue. Midline rostral diencephalic tissue induced Islet-1<sup>+</sup>/SC1<sup>+</sup> neurons and *Nkx 2.1*<sup>+</sup> cells in telencephalic level explants (Fig. 13C and data not  
30 shown). In contrast, ventral midline diencephalic tissue isolated at the level of the infundibulum, a region which does not express *vhh-1/shh* (Fig. 8, 9Ai, Echelard et al., 1993), did not induce Islet-1<sup>+</sup> cells in these explants (data not shown).

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Finally, applicants tested whether the inductive activity of neural tissue sources of *vhh-1/shh* differed according to their rostrocaudal position. Conjugates were formed between floor plate tissue, a caudal source of *vhh-1/shh*, and telencephalic level neural plate explants. Floor plate tissue was effective in inducing Islet-1<sup>+</sup>/SC1<sup>+</sup> neurons (Fig. 13D, E) and Nkx 2.1<sup>+</sup> cells (Fig. 13F) in telencephalic level neural plate explants. Moreover, the Islet-1<sup>+</sup> neurons did not express Lim-1 (data not shown) indicating that they have a characteristic telencephalic phenotype. Thus, the specific identities of ventral neurons that are induced by neural sources of *vhh-1/shh* appear to depend on rostrocaudal restrictions in the response properties of neural plate cells and not on the axial level of origin of the inducing tissue.

#### EXPERIMENTAL DISCUSSION

A vertebrate homolog of the *Drosophila hedgehog* gene, *vhh-1/shh*, is expressed by the notochord and floor plate and can mimic the ability of these two midline cell groups to induce motor neuron differentiation (Roelink et al., 1994). *vhh-1/shh* has, therefore, been implicated in the induction of ventral neuronal types at caudal levels of the neuraxis. The present studies and previous analyses show that *vhh-1/shh* is expressed by cells in the region of the diencephalon rostral to the floor plate and also in the ventral telencephalon (Echelard et al., 1993; Krauss et al., 1993; Chang et al., 1994; Roelink et al., 1994), raising the question of whether *vhh-1/shh* also participates in the induction of ventral neurons in the forebrain.

Applicants have found that *vhh-1/shh* induces the differentiation of ventral neuronal cell types

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characteristic of the diencephalon and telencephalon in regions of the neural plate that normally give rise to these two subdivisions of the forebrain. The LIM homeodomain protein Islet-1, an early marker of motor neuron differentiation at caudal levels of the neural tube, is also induced by vhh-1/shh early in the differentiation of these ventral diencephalic and telencephalic neurons. Islet-1<sup>+</sup> neurons, however, have distinct regional identities that appear to be constrained by the axial level of origin of cells within the neural plate. Thus, a single inducing molecule, vhh-1/shh, may participate in the differentiation and diversification of ventral neuronal cell types along the entire rostrocaudal extent of the neural tube acting on neural plate cells of predetermined rostrocaudal character.

One limitation of the present studies is that the eventual identity and function of the embryonic forebrain neurons induced by vhh-1/shh is not known. In the adult forebrain, Islet-1 is expressed by diencephalic neurons in the suprachiasmatic and arcuate nuclei of the hypothalamus, in the zona incerta, the septal and thalamic reticular nuclei and by basal telencephalic neurons (Thor et al., 1991). It is likely, therefore, that neurons in these ventral forebrain nuclei represent the mature derivatives of the Islet-1<sup>+</sup> neurons that are induced by vhh-1/shh at prospective forebrain levels of the neural plate.

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#### vhh-1/shh as a Direct Inducer of Ventral Neurons

In neural plate explants obtained from spinal cord and rhombencephalic levels, vhh-1/shh induces motor neurons (Fig. 10, 11; Roelink et al., 1994). Since floor plate

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cells are also induced under these conditions, this observation does not resolve whether motor neuron differentiation results from the activity of vhh-1/shh directly or from the actions of a distinct floor plate-derived inducing molecule. In diencephalic level explants, only approximately 35% of cells were induced to differentiate into Islet-1<sup>+</sup> neurons and it is possible that diencephalic cells with specialized midline signalling properties are also induced in these explants. Thus, at diencephalic as well as at more caudal levels, vhh-1/shh could induce the production of a distinct midline-derived factor that is responsible for the generation of ventral neurons. In contrast, in telencephalic level neural plate explants, vhh-1/shh caused virtually all cells to differentiate into Islet-1<sup>+</sup> neurons of telencephalic character. This result provides strong evidence that vhh-1/shh can induce ventral neurons by an action on neural plate cells that is independent of the induction of specialized midline cells.

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#### Early Restriction in the Rostrocaudal Character of Neural Plate Cells

Embryological studies have provided evidence that the rostrocaudal and dorsoventral character of cells within the neural plate and neural tube is controlled by independent patterning systems (Doniach et al., 1992; Ruiz i Altaba, 1992; Jessell and Dodd, 1992; Smith, 1993). The early rostrocaudal character of neural cells appears to be established prior to the definition of cell identity along the dorsoventral axis of the neural tube (Roach, 1945; Jacobson, 1964; Simon et al., 1995). Applicants' in vitro results support this idea and in addition show that the rostrocaudal character of neural cells that has been defined at the neural plate stage is

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maintained in vitro, both in the absence and presence of ventralizing signals mediated by vhh-1/shh. Thus, an early and stable restriction in the potential of cells located at different rostrocaudal positions within the neural plate appears to define the repertoire of ventral neuronal cell types that can be generated upon exposure of cells to vhh-1/shh.

The signals that establish which the early rostrocaudal character of neural plate cells have not been identified. However, studies in several vertebrate species have provided evidence that the action of these signals subdivides the neural tube along its rostrocaudal axis, into discrete domains or segments (Vaage, 1969; Figdor and Stern, 1993; Lumsden and Keynes 1989). Many or all of these segmental domains coincide with the boundaries of expression of transcription factors (Rubenstein et al., 1994; Macdonald et al., 1994; Papalopulu, 1994). The intrinsic restriction in the potential fates of neural plates cells might, therefore, be established by the early and regionalized expression of transcription factors that later reveal segmental subdivisions of the neural tube.

#### Homeobox Gene Expression and a Common Program for the Generation of Ventral Neurons

The detection of Islet-1 in ventral neuronal cell types generated at many different positions along the rostrocaudal extent of the neural tube suggests that the expression of this gene is more closely associated with the differentiation of neurons of ventral character than with the generation of any specific class of ventral neuron. However, at rhombencephalic and mesencephalic levels, the differentiation of serotonergic and

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dopaminergic neurons can be induced by the notochord and floor plate but these neurons do not express Islet-1 (Yamada et al., 1991; Hynes et al., 1995 and applicants' unpublished observations). Thus, although Islet-1 expression is a prominent marker of ventral neuronal differentiation, its expression is not always associated with the generation of ventral neuronal cell types that depend on notochord- and floor plate-derived signals.

Nevertheless, the expression of Islet-1 by many distinct classes of ventral neurons raises the possibility that elements of the response of neural plate cells to *vhh-1/shh* may be conserved along the rostrocaudal axis. In support of this, members of the *Nkx 2* family of homeobox genes, notably *Nkx 2.1* and *Nkx 2.2* are expressed in the ventral neural tube at all rostrocaudal levels, in a domain that overlaps closely with that of *vhh-1/shh* (Price et al., 1992; Lazzaro et al., 1991; Rubenstein et al., 1994). Moreover, at forebrain levels the expression of *NKX 2.1*, is induced by *vhh-1/shh*. Thus, the *Nkx 2* and Islet-1 homeodomain proteins might represent elements of a common *vhh-1/shh*-response program that is activated in neural plate cells independent of their rostrocaudal position.

#### The Source of Signals that Induce Ventral Neurons In Vivo

Cells in the floor plate and at the ventral midline of the rostral diencephalon represent likely neural sources of signals involved in the induction of ventral neurons in vivo. However, the notochord and prechordal plate express *vhh-1/shh* (Riddle et al., 1993; Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994), and could, therefore, also participate in the induction of ventral neuronal cell types. Indeed, in vitro studies of

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motor neuron differentiation at spinal cord levels have provided evidence that the signals responsible for induction of the earliest-born motor neurons derive from the notochord, with the floor plate acquiring a more prominent role in the differentiation of motor neurons only at larger stages (Yamada et al., 1993).

At telencephalic levels, however, the induction of ventral neurons is unlikely to depend on signals from the axial mesoderm, since the region of the neural plate that gives rise to the floor of the telencephalon is never contacted by prechordal plate mesoderm (Couly and Le Douarin, 1987; Placzek, M., unpublished data). Moreover, Islet-1<sup>+</sup> neurons of the ventral forebrain are not specified until HH stage 14 (Muhr, unpublished data). It is possible that telencephalic Islet-1<sup>+</sup> neurons or their precursors migrate from the rostral diencephalon into the telencephalon. Alternatively, neural tissue might be a source of *vhh-1/shh* involved in the induction of the Islet-1<sup>+</sup> neurons in the ventral telencephalon. This neural source is unlikely to derive from the telencephalon itself, however, since *vhh-1/shh* is not expressed by cells at the floor of the telencephalon until HH stages 17-18, coincident with the appearance of telencephalic Islet-1<sup>+</sup> neurons.

Cells at the ventral midline of the rostral diencephalon could provide a source of signals that induce Islet-1<sup>+</sup> neurons in the ventral telencephalon since they express *vhh-1/shh* at HH stage 9. Consistent with this, in vitro studies show that midline rostral diencephalic cells that express *vhh-1/shh* can induce Islet-1<sup>+</sup> neurons in telencephalic regions of the neural plate. It remains possible that rostral diencephalic cells secrete other factors that cooperate with *vhh-1/shh* to define the



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number and diversity of ventral cell types generated at the floor of the telencephalon. This might account for the difference between in vitro results, in which vhh-1/shh induced virtually all cells in telencephalic neural plate explants to differentiate into Islet-1<sup>+</sup> neurons, and in vivo analyses showing a sparse scattering of Islet-1<sup>+</sup> neurons at the ventral midline of the telencephalon. Alternatively, expression of vhh-1/shh in COS cells could expose telencephalic neural plate explants to a higher level of inducer than is provided in vivo and in vitro by rostral diencephalic cells. Independent of the identity of the endogenous diencephalic inducers, these observations suggest that the differentiation of neurons in the ventral telencephalon is normally dependent on signals provided in a planar manner by midline cells of the rostral diencephalon.

Taken together, these studies implicate vhh-1/shh in the induction of ventral neuronal types along the entire rostrocaudal extent of the embryonic central nervous system. Several prominent classes of neurons that are depleted in neurodegenerative diseases derived from ventrally-located progenitors at different axial levels of the neural tube: motor neurons at spinal levels, dopaminergic neurons at mesencephalic levels and striatal and basal forebrain neurons at telencephalic levels. Since vhh-1/shh appears to direct the ventral neuronal fates of progenitor cells during embryogenesis, the protein might exert a similar activity on neuronal progenitors present in the adult (Reynolds and Weiss, 1992) and thus could repopulate the central nervous system with classes of ventral neurons depleted in neurodegenerative disease.

#### 35     EXPERIMENTAL PROCEDURES

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Animals

Fertilized white leghorn chicken eggs were obtained from Agrisera AB, Sweden. Chick embryos were staged according to Hamburger and Hamilton (1951). Time mated mouse embryos (C57/bl) were obtained from the animal facility, University of Umea.

Neural Plate Fate Mapping

Glass micropipettes with fine tip diameters were filled with Di-I (1, 1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (Molecular Probes; 2.5 mg ml<sup>-1</sup> in DMSO). 1-5 nl of Di-I was injected into defined regions of the neural plate of HH stage 6 chick embryos using an automated microinjection system. Embryos were permitted to develop until HH stages 10/11 or stage 15 and the neural tube was then isolated. The position of Di-I labeled cells was mapped using phase contrast and epifluorescence optics and compared to the fate map of Couly and Le Douarin (1987) or assessed using morphological landmarks.

In Situ Hybridization and Immunohistochemistry

In situ hybridization analysis of mRNA expression of cryostat sections was performed using a 1.7 kb digoxigenin-labeled chick *vhh-1/shh* riboprobe (T. Lints and J. Dodd, unpublished data) essentially as described (Schaeren-Wiemers and Gerfin-Moser, 1993). Sections processed for in situ hybridization were washed for 4x10 minutes in Tris-buffered saline containing 0.1% Triton X-100 (TBST), blocked in TBST containing 10% normal goat serum and incubated with primary rabbit anti-Islet-1 antibodies (1:250) overnight at 22°C. Islet-1 was

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detected using an avidin/biotin-complex as described (Thor et al., 1991), except that the incubation times were doubled and the slides were mounted in a glycerol-based mounting media. Whole-mount in situ hybridization was performed as described (Francis et al., 1994).

Islet-1 was detected using rabbit and anti-Islet-1 antibodies (Thor et al., 1991; Ericson et al., 1992) or MAb 4D5 (Roelink et al., 1994). Lim-1 (Taira et al., 1992) was detected with MAb 4F2 which also recognizes Lim-2 (Tsuchida et al., 1994). In situ hybridization studies indicate that the patterns of expression of *Lim-1* and *Lim-2* mRNAs in embryonic forebrain are similar (data not shown). Thus, applicants cannot resolve whether Lim-1 and/or Lim-2 are expressed by individual cells labeled with MAb 4F2. This does not affect the use of the antibody to distinguish Islet-1<sup>+</sup> neurons at different forebrain levels. The SC1 glycoprotein was detected with MAb SC1 (Tanaka and Obata, 1984), the homeodomain protein Nkx-2.1 with rabbit and anti-Nkx-2.1 antibodies (Lazzaro et al., 1991), the floor plate marker FP1 with MAb FP1 (Yamada et al., 1991), anti-nestin with antisera 129/130 (Dahlstrand et al., 1992), anti-acetylated  $\beta$  tubulin was detected using the monoclonal antibody T6793 (Sigma immunochemicals) and neuronal cytoplasm using the anti-cyn-1 antibody (S.B. Morton and T. Jessell, unpublished). The number of Islet-1 and Lim-1 cells in explants was determined by sectioning explants and counting the number of labeled cells in every fifth section. The total number of cells in these sections was determined by nuclear labeling DAPI (Boehringer Mannheim). Other markers used were analyzed by whole-mount immunohistochemistry as described (Yamada et al., 1993).

#### Isolation and Culture of Neural Plate Explants

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Eggs were incubated at 38°C in a humidified incubator. HH stage 6 embryos were collected in L15 (GIBCO-BRL) medium at 4°C, incubated in dispase solution (Boehringer Mannheim, 2 mg/ml in L15) at 22°C for 4 minutes and transferred into L15 at 4°C containing 5% heat-inactivated fetal calf serum. Embryos were washed three times in L-15 and neural tissue was separated from adherent mesoderm and endoderm. Neural plate explants corresponding to presumptive telencephalic, diencephalic and rhombencephalic regions were dissected using tungsten needles. Floor plate from HH stage 25 chick embryos was isolated as previously described (Yamada et al., 1993). Midline rostral diencephalic tissue expressing *vhh-1/shh* (Echelard et al., 1993) was dissected from E11 mouse embryos. Neural plate explants were cultured for 60-66 hours in contact with COS cell aggregates, floor plate fragments or diencephalic tissue in three-dimensional collagen gels (Vitrogen 100, Celtrix Laboratories) in 600 µl of OPTIMEM-1 supplemented with N2-supplement, human fibronectin (5 µg/ml) and penicillin/streptomycin (media and additives from GIBCO-BRL, Inc.).

#### Expression of rat *vhh-1* in COS Cells

COS cells were grown until 90% confluency and transfected with 1 µg of DNA per 35 mm dish with 12 µg/ml lipofectamine reagent (GIBCO BRL) in Dulbecco's modified Eagle's medium (DMEM). After a 5 hour incubation, medium was replaced with DMEM containing 10% FCS and cells were incubated for additional 18 hours. COS cells were then dissociated using PBS containing 2mM EDTA, pelleted and resuspended in DMEM containing 10% FCS and antibiotics. Cell aggregates were made by hanging a 20 µl drop containing about 1000 cells on the lid of a tissue culture plate as described (Roelink et al., 1994). After

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24 hours, aggregates were washed in OPTIMEM-1 and placed in contact with chick neural plate explants.

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Third Series of Experiments

During vertebrate development, the generation of cell types in the ventral half of the neural tube depends on signals provided by axial mesodermal cells of the notochord (1-6). The notochord appears to be the source of a contact-dependent signal that induces floor plate cells at the ventral midline of the neural tube, and a diffusible signal that induces motor neurons independent of floor plate differentiation (2,7,8,9). Floor plate cells subsequently acquire both these inducing activities (5,7,9). Sonic hedgehog (shh)/vhh-1, a vertebrate homolog of the secreted glycoprotein encoded by the *Drosophila* gene, *hedgehog* (10,11), is expressed by the notochord and floor plate at the time that these midline cell groups exhibit their inductive activities (12-16). Shh/vhh-1 can induce ectopic floor plate differentiation in the neural tube in vivo (13-15) and in neural plate explants in vitro (15) suggesting that it participates normally in floor plate induction. Whether the notochord- and floor plate-derived diffusible factor that induces motor neurons is also shh/vhh-1, however, remains unclear. Motor neurons are induced in neural plate explants grown in contact with cells that express shh/vhh-1 (15), but this could reflect the activity of a distinct factor secreted by the floor plate cells that are also induced in these explants. Applicants show here that: i) COS cells transfected with shh/vhh-1 acquire a diffusible activity that is sufficient to induce motor neurons in neural plate explants in the absence of floor plate differentiation, ii) that shh/vhh-1 itself can act on cells in neural plate explants to induce, independently, motor neurons and floor plate cells. These results suggest that shh/vhh-1 provided by the notochord normally initiates the differentiation of motor

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neurons as well as floor plate cells in the neural tube of vertebrate embryos.

5 Floor plate and motor neuron differentiation was monitored in explants derived from the intermediate region of the neural plate of Hamburger Hamilton (HH) stage 10 chick embryos (8) using immunocytochemical and reverse transcription-polymerase chain reaction (RT-PCR) assays. Floor plate differentiation was assessed  
10 primarily by expression of the winged helix transcription factor HNF3 $\beta$  (Table 4). HNF3 $\beta$  is an early marker of floor plate differentiation in vivo (17,18) and its transcription in neural plate cells in vitro is a direct response to notochord-derived signals since it can occur  
15 in the absence of protein synthesis (17). Moreover, misexpression of HNF3 $\beta$  in the neural tube is sufficient to trigger ectopic floor plate cells (19,20) which, in turn, can induce ventral neurons in adjacent dorsal regions of the neural tube (19). Thus, HNF3 $\beta$  expression  
20 provides an early and reliable indicator of floor plate differentiation. As an independent marker of floor plate differentiation, applicants monitored expression of mRNA encoding the chemoattractant, Netrin-1 (Table 4). Motor neuron differentiation was assessed by expression of the  
25 LIM homeodomain proteins Isl-1 and Isl-2 (21), by coexpression of SC1 with Isl-1 and Isl-2 and by expression of *Isl-1*, *Isl-2* and *choline acetyltransferase* (*ChAT*) mRNAs (Table 4).

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Table 4. Markers of Floor Plate and Motor Neuron Differentiation in Chick Neural Plate Tissue.

	<u>Floor Plate Cells</u>	<u>Reference</u>	<u>Motor Neurons</u>
5	<u>Reference</u>		
	HNF3 $\beta$	(18,19)	Isl-1/SC1
	(5,8,20,34)		
	Netrin-1	(32,33)	Isl-2 (20)
			ChAT (8)
10	Neural plate explants (8) that were grown alone in vitro for 36 h did not express floor plate or motor neuron markers (Fig. 14A, E, F, Table 5A). In contrast, neural plate explants grown in contact with notochord for 36 h		
15	expressed HNF3 $\beta$ mRNA and protein (Fig. 14B, D, E) and Netrin-1 mRNA (Fig. 14E) indicating the differentiation of floor plate cells. The same explants contained cells that expressed Isl-1 and/or Isl-2 (termed Isl+ cells) in combination with SC1 (Fig. 14B, C, D, F), and Isl-1, Isl-		
20	2 and ChAT mRNAs (Fig. 14F) indicating the differentiation of motor neurons. To separate experimentally, the motor neuron- and floor plate-inducing activities of the notochord, applicants prevented contact between the notochord and neural plate		
25	explants by interposing a membrane filter. In the absence of contact, the notochord induced motor neuron differentiation (Fig. 14G, H), albeit less effectively, as assessed by the number of Isl+ neurons (Table 5A).		



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**Table 5. Induction of Floor Plate and Motor Neuron Markers in Neural Plate Explants.**

	(Number of explants)	HNF3 $\beta$ cells/explant	Isl <sup>+</sup> cells/ explant	
	<b>A. Induction by notochord</b>			
10	neural plate	0	<1	0
	notochord + neural plate	286 $\pm$ 40	215 $\pm$ 8	5
15	notochord/filter/ neural plate	0	38 $\pm$ 10	1
	<b>B. Induction by shh/vhh-1</b>			
20	antisense vhh-1 + neural plate	0	0	2
25	sense vhh-1 + neural plate	100 $\pm$ 23	182 $\pm$ 28	9
	sense vhh-1/filter/ neural plate	0	47 $\pm$ 8	0
30	sense vhh-1/collagen/ neural plate	0	49 $\pm$ 5	9

Neural plate explants were grown for 36 h with the notochord (A) or vhh-1-transfected COS cells (B) either in contact (indicated by + sign) or separated by membrane filters or by a strip of collagen gel (indicated by //). Values are mean  $\pm$  s.e.m.

In contrast, the notochord did not induce floor plate differentiation across a filter, as assessed by the absence of HNF3 $\beta$  expression at 24 h (data not shown) or 36 h (Fig. 14G, Table 5A). These results extend previous observations (7,8) in that they show that a notochord-derived diffusible factor can induce motor neurons in the absence of floor plate differentiation within the same neural plate explant.

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To examine whether *shh/vhh-1* can mimic the contact-dependent and diffusible activities of the notochord, applicants grew neural plate explants for 36 h either in contact with, or separated from, COS cells transfected with sense or antisense cDNA constructs encoding the rat *shh* homologue, *vhh-1* (15). Neural plate explants grown in contact with COS cells transfected with sense *vhh-1* contained both floor plate cells, assessed by expression of HNF3 $\beta$  (Fig. 15A, G lane 1, Table 4) and Netrin-1 (Figure 15G lane 1) and motor neurons, assessed by expression of Isl<sup>+</sup>/SCl<sup>+</sup> neurons (Fig. 15A, B, C), Isl-1 and ChAT (Fig. 15H lanes 1). Neural plate plants grown in the absence of contact with COS cells transfected with sense *vhh-1* did not express (Fig. 15D, G lane 3) or Netrin-1 (Fig. 15G lane 3). In contrast, motor neuron differentiation was induced in the absence of contact, as assessed by expression of Isl<sup>+</sup>/SCl<sup>+</sup> neurons (Fig. 15D, E, F, Table 5B), Isl-1 and ChAT (Fig. 15H lanes 1). Neural plate explants grown in the absence of contact with COS cells transfected with sense *vhh-1* did not express HNF3 $\beta$  (Fig. 15D, G lane 3) or Netrin-1 (Fig. 15G lane 3). Medium conditioned by *vhh-1*-transfected COS cells does not induce floor plate or motor neuron differentiation in neural plate explants (15). In the present experiments, the differentiation of motor neurons in neural plate explants grown at a distance from *vhh-1*-transfected COS cells may result from the provision of a higher concentration or of a constant source of *shh/vvh-1*. COS cells transfected with antisense *vhh-1* did not induce floor plate or motor neuron differentiation under any condition (Fig. 15G, H lanes 2 and 4 and data not shown). Expression of *vhh-1*, therefore, confers COS cells with a contact-dependent floor plate-inducing activity and a diffusible motor neuron inducing-activity that does not elicit floor plate differentiation. The most likely

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explanation of these results is that *shh/vhh-1* itself mediates both these activities. A diffusible form of *shh/vhh-1* has also been implicated in the introduction of *Pax-1* expression in segmental plate mesoderm (22).

5 To examine whether *shh/vhh-1* can itself induce motor neuron differentiation, applicants transfected *vhh-1* expression constructs directly into cells within neural plate explants. Neural plate explants assayed 48 h after  
10 transfection with *vhh-1* expressed *HNF3 $\beta$* , *Netrin-1*, *Isl-1* and *Isl-2* (Fig. 16A). *Shh/vhh-1* is, therefore, sufficient to induce floor plate and motor neuron differentiation in neural plate explants. To determine whether the induction of motor neurons in neural plate  
15 explants transfected with *vhh-1* occurs independently of floor plate differentiation, applicants analyzed the time course of expression of *HNF3 $\beta$*  and *Isl-1*. Expression of *Isl-1* in neural plate explants transfected with *vhh-1* was first detected after ~22 h and either preceded (Fig.  
20 16Bii) or occurred coincidentally (Fig. 16Bi) with that of *HNF3 $\beta$* , depending on the particular experiment. Thus, motor neuron differentiation in neural plate explants transfected with *vhh-1* occurs prior to or synchronously with floor plate differentiation. *Shh/vhh-1*, therefore,  
25 appears to act on neural plate cells to induce the differentiation of motor neurons in a manner that does not require the prior differentiation of floor plate cells (15). Previous studies in chick embryos have shown that cells in lateral regions of the neural plate are  
30 exposed to a motor neuron-inducing signal from the notochord prior to the differentiation of floor plate cells (8). The early expression of motor neuron markers in neural plate explants transfected with *vhh-1* provides evidence that this signal is *shh/vhh-1*.

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Taken together, applicants' results suggest that the ability of the notochord to induce floor plate differentiation in a contact-dependent manner and motor neuron differentiation via a diffusible factor can be attributed to independent activities of *shh/vhh-1*. They do not exclude that the induction of motor neurons by *shh/vhh-1* involves the synthesis by neural plate cells of a distinct secreted factor, in a manner similar to the proposed involvement of *dpp* and *wg* as mediators of the long-range patterning activities of hedgehog in the imaginal disc epithelia of *Drosophila* (23-25). In the neural tube, however, vertebrate homologs of *dpp* (BMP proteins) and *wg* (wnt proteins) have dorsalizing actions (26, 27), and are, therefore, unlikely to act as mediators of the ventralizing actions of *shh/vhh-1*.

The mechanism by which *shh/vhh-1* induces the differentiation of floor plate cells and motor neuron remains unclear. *Drosophila* and vertebrate hedgehog proteins undergo autoproteolysis to generate an amino-terminal fragment (N) which is associated with the cell surface and a carboxy-terminal (C) fragment which is freely diffusible (28). The induction of floor plate and motor neuron differentiation could, therefore, result from distinct biological activities that reside in the processed N and C fragments of *shh/vhh-1* (Fig. 17A). Alternatively, floor plate and motor neuron fates could be specified by different concentrations of a single *shh/vhh-1* fragment (Fig. 17B), in a manner similar to that proposed for TGF $\beta$ -related proteins in the patterning of mesodermal tissues in vertebrate embryos (29-31).

#### Materials and Methods

Intermediate neural plate explants were dissected from

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the caudal region of the neural plate of Hamburger-Hamilton (36) (HH) stage 10 chick embryos as described (8). Notochord explants were dissected after dispase treatment from the caudal region of HH stage 10 chick embryos. Conjugates between notochord and neural plate explants were prepared in collagen gels. When required, notochord and neural plate explants were separated by Nucleopore polycarbonate (pore size 0.1  $\mu$ m, COSTAR) or dialysis membrane (Spectrum, Spectra/Por membrane MW cut off: 50,000) filters. Explants were grown in defined medium as described (8).

Detection of Neural Markers: HNF3 $\beta$  was detected with rabbit antibodies(18,19), Isl-proteins were detected by antibodies that recognize both Islet-1 and Islet-2 (Isl<sup>+</sup> cells), or by Isl-1-specific or Isl-2-specific monoclonal antibodies (20,34) (Morton, S., unpublished data). The SC1 glycoprotein was detected with MAb SC1 (35). Neural plate explants were fixed with 4% paraformaldehyde at 4°C for 1-2 h and washed with phosphate-buffered saline (pH 7.4) at 4°C for 1-2 h. Explants were incubated with primary antibodies overnight at 4°C, then with FITC-conjugated goat anti-mouse IgG (Boehringer Mannheim) or Texas red-conjugated goat anti-rabbit Ig G (Molecular Probes) for 1-2 h at 22°C. The explants were then washed and mounted on slides in 50% glycerol: 50% 0.1 M carbonate buffer, pH 9.0 containing paraphenylene diamine (0.4 mg/ml). Explants were examined on a Zeiss Axiophot microscope equipped with epifluorescence optics. Optical sectioning of explants was performed on a Bio-Rad MRC-500 confocal microscope.

Competitive PCR analysis: RT-PCR analysis was performed essentially as described (8). Total RNA was extracted from 10-20 explants cultured in collagen gel with 5  $\mu$ g of

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glycogen as carrier (37). An internal standard for competitive PCR analysis was prepared by deleting (in *HNF3 $\beta$* , *Isl-1*) or inserting (in *Isl-2*, *Netrin-1*, *ChAT*) a 200-300 bp fragment within the sequence to be amplified.

5 Plasmid DNAs were linearized and transcribed in vitro to prepare sense-oriented RNA. 100 fg of competitive template RNA was added to the total RNA of each sample and was reverse transcribed using MoMLV-RT (Gibco BRL). One tenth of each reaction product was subjected to PCR

10 using specific primers flanking the deleted or inserted site of each clone. *HNF3 $\beta$*  : 5'-TCA CCA TGG CCA TCC AGC AGT CG and 5' -CAG CAG GTG CTG CGC TGG AGA GG, *Netrin-1* : 5'-TGG GCA GCA CCG AGG AC and 5'-CCT TCC ATC CCT CAA TA, *Isl-1*: 5'-TCA AAC CTA CTT TGG GGT CTT A and 5'-ATC

15 GCC GGG GAT GAG CTG GCG GCT, *Isl-2*: 5'-TGC TGA ACG AGA AGC AG and 5'-TGG TAG GTC TGC ACC TCC A, *ChAT* : 5'-TCC ATA CGC CGA TTT GAT GAG GGC and 5'-CTA TTG CTT GTC AAA TAG GTC TCA. Each PCR cycle was at 94°C for 1 min., 54°C for 1 min. and 72°C for 1 min. Twenty two cycles were

20 used for amplifying *Isl-2*, *Isl-1*, *HNF3 $\beta$*  and *Netrin-1* and twenty cycles for *ChAT*. The PCR products were detected by Southern Blot hybridization with <sup>32</sup>P-labeled DNA probes. Blots are aligned such that the tissue-derived band is above the internal standard. Sizes of tissue-

25 derived PCR bands are: *HNF3 $\beta$*  : 510 bp, *Netrin-1* : 232 bp, *Isl-1* : 427 bp, *Isl-2* : 304 bp, *ChAT*: 283 bp.

COS cell transfections: Transfections with sense or antisense vhh-1 expression plasmids were performed as

30 described (15). Briefly, 1 ug of DNA and 12 ug/ml or Lipofectamine (GIBCO BRL) in Dulbecco's modified Eagles medium (DMEM) supplemented with 1% glutamine was added to the 80-90% confluent COS cells in 35mm dishes. After 5 h of incubation, the transfection reaction was stopped by

35 replacing the medium with DMEM-supplemented with 10% calf

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serum. Induction assays were carried out after 36 h of incubation. For induction of floor plate cells and motor neurons by vhh-1-transfected COS cells, intermediate neural plate explants were placed on a monolayer of transfected COS cells, embedded in the collagen gel and cultured for 36 h in F12/N3 medium. To prepare transfilter assays, intermediate neural plate explants were separated from COS cells by a polymerized collagen gel, by Nucleopore Polycarbonate filter or by dialysis membrane. (See Fig. 14 legend.)

Neural Plate Transfections: CMV- or RSV-LTR-based vhh-1 expression plasmids were transfected directly into intermediate neural plate explants using Lipofectamine (GIBCO BRL). 400 ng of DNA and 2 ug of Lipofectamine were mixed in 100  $\mu$ l of F12/N3 and added to neural plate explants. The explants were incubated for 5 h, rinsed and cultured in collagen gels as described (8). In experiments on vhh-1-transfected explants, 28 cycles of amplification were used on 1/100th of the tissue-derived cDNA product. The viability of neural plate explants subjected to the transfection protocol was impaired (data not shown). Applicants therefore supplemented the culture medium with neurotrophin 3 (NT3; 10 ng/ml: Genentech, Inc.) which has no floor plate or motor neuron-inducing activity (Fig. 14A and data not shown), but which enhances the number of motor neurons that differentiate in dissociated neural tube cultures (38).

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Fourth Series of Experiments

Intercellular signaling molecules of the vertebrate hedgehog family and transcription factors of the winged-helix family have been implicated in floor plate development. Applicants have examined the consequences of misexpressing the vertebrate *hedgehog* gene *vhh-1* (*sonic hedgehog*, *shh*) and the winged-helix gene *HNF-3 $\beta$*  in the neural plate and neural tube of frog embryos. Misexpression of either of these genes induces floor plate differentiation at ectopic locations. However, ectopic floor plate induction in response to both *vhh-1* and *HNF-3 $\beta$*  was temporally and spatially restricted. At neural plate stages, ectopic floor plate differentiation was not detected. After neural tube closure, ectopic floor plate differentiation, was detected, but was restricted predominantly to the dorsal region of the neural tube. The ability of winged-helix and vertebrate *hedgehog* genes to induce floor plate differentiation *in vivo* may, therefore, be constrained by additional signals that specify the time and position of floor plate differentiation.

Introduction

Cells at the midline of the vertebrate embryo act as organizing centers, providing local signals that control the pattern of mesodermal and neural cell differentiation. Axial mesodermal cells of the notochord influence the pattern of cell types generated along the dorsoventral (D-V) axis of the neural tube. In chick embryos, notochord grafts can induce the differentiation of floor plate cells and motor neurons at ectopic locations in the neural tube (van Straaten et al., 1988; Placzek et al., 1990, 1993; Yamada et al., 1991, 1993). Inversely, removal of the notochord prevents the

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differentiation of floor plate cells and motor neurons (van Straaten and Hekking, 1991; Placzek et al., 1990; Yamada et al., 1991; Ericson et al., 1992; Goulding et al., 1993; but see Artinger and Bronner-Fraser, 1993).

5 In mouse, mutations that eliminate the notochord also prevent floor plate and motor neuron differentiation (Bovolenta and Dodd, 1991; Ang and Rossant, 1994; Weinstein et al., 1994). Similarly, in frog embryos the

10 differentiation of floor plate cells and motor neurons is inhibited if notochord formation is prevented (Clarke et al., 1991) or if the notochord develops at a distance from the neural ectoderm (Ruiz i Altaba, 1994). The organizer region and the floor plate can mimic the inductive actions of the notochord (Wagner et al., 1990;

15 Yamada et al., 1991, 1993; Hatta et al., 1991; Placzek et al., 1993), raising the possibility that signalling molecules expressed by these three midline cell groups may be conserved (Ruiz i Altaba and Jessell, 1993).

Intercellular signalling molecules and transcription

20 factors that appear to participate in floor plate development have been identified. A vertebrate homolog of the *Drosophila* gene *hedgehog*, *vhh-1/shh*, encodes a putative secreted protein and is expressed by cells in the organizer region, the notochord and the floor plate

25 at the time that these cell groups exhibit their inductive activities (Riddle et al., 1993; Krauss et al., 1993; Echelard et al., 1993; Roelink et al., 1994). The same three cell groups also express members of the winged-helix (HNF-3/fork head) family of DNA-binding

30 transcription factors (Lai et al., 1990; 1991; Weigel and Jäckle, 1990; Clark et al., 1993): *Pintallavis* (also known as *XFKH1* or *XFD1/1'*), *HNF-3 $\beta$*  (also known as *axial*) and *HNF-3 $\alpha$*  (also known as *XFKH2*) (Ruiz i Altaba and Jessell, 1992; Dirksen and Jamrich, 1992; Knöchel et al.,

35 1992; Ruiz i Altaba et al., 1993b; Bolce et al., 1993;

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Sasaki and Hogan, 1993; Ang et al., 1993; Monaghan et al., 1993; Strähle et al., 1993). In frog embryos, *Pintallavis* appears to be the functional homolog of mammalian HNF-3 $\beta$  at gastrula stages: *Pintallavis* is expressed transiently in the organizer, notochord and floor plate whereas HNF-3 $\beta$  does not appear until neurula stages.

Evidence for the involvement of vertebrate *hedgehog* and winged-helix genes in neural patterning has derived from an analysis of cell differentiation in the neural tube after misexpression of these genes. Misexpression of *vhh-1/shh* in mouse, frog or zebrafish embryos leads to the ectopic expression of floor plate markers in the neural tube *in vivo* (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994) and *vhh-1* expression in COS cells induces floor plate and motor neuron differentiation in rat and chick neural plate explants *in vitro* (Roelink et al., 1994). Misexpression of *Pintallavis* in frog embryos also leads to the appearance of floor plate markers in dorsal regions of the neural tube and to a reduction in the number of dorsal sensory neurons (Ruiz i Altaba and Jessell, 1992; Ruiz i Altaba et al., 1993a). Similarly, transgenic mice that express HNF-3 $\beta$  throughout the midbrain express floor plate markers ectopically (Sasaki and Hogan, 1994). Moreover, mice in which the HNF-3 $\beta$  gene has been inactivated by targeted mutation display a perturbation in node development, lack a notochord and exhibit a loss of floor plate cells and motor neurons (Weinstein et al., 1994; Ang and Rossant, 1994). These results suggest that the vertebrate *hedgehog* gene *vhh-1/shh* and members of the winged-helix transcription factor family participate in the specification of midline fates and in the patterning of the neural tube by axial midline cell groups.

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Clarification of the mechanisms by which vertebrate *hedgehog* and winged-helix genes normally act in midline neural plate and neural tube cells requires the determination of their sufficiency in eliciting floor plate differentiation. To address this issue applicants have analyzed, in parallel, the actions of *vhh-1/shh* and HNF-3 $\beta$  on neural cell patterning in frog embryos in vivo. Applicants show here that *vhh-1* and HNF-3 $\beta$  can each activate expression of the other gene and that both genes can cause ectopic floor plate differentiation in the neural tube. However, applicants have found marked temporal and spatial constraints on the ability of *vhh-1* and HNF-3 $\beta$  to induce ectopic floor plate cells. These findings suggest that the ability of *vhh-1*, *Pintallavis* and HNF-3 $\beta$  to promote floor plate differentiation in vivo is constrained by additional factors.

#### EXPERIMENTAL RESULTS

##### Isolation and Pattern of Expression of Frog *vhh-1*

To examine the effects of deregulated expression of the endogenous *vhh-1* gene in frog embryos, applicants cloned several *Xenopus laevis* *vhh-1* cDNAs (see Experimental Procedures) one of which contained a ~1.4kb open reading frame, encoding a protein with ~70% identity *vhh-1/shh* genes identifies in other vertebrate species (Genbank accession number L35248).

The pattern of expression of *vhh-1* in early frog embryos was analyzed by in situ hybridization and compared to that of the winged-helix genes *Pintallavis* and HNF-3 $\beta$  and to the homeobox gene *goosecoid*. Expression of *vhh-1* mRNA in frog embryos was first detected at early gastrula stages (stage 10+) in cells within the medial region of the dorsal blastopore lip (Fig 18A, stage 10 and not

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shown) and occurred after that of *Pintallavis* and *gooseoid* (Figs. 18B, C; Cho et al., 1991; Dirksen and Jamrich, 1992; Ruiz i Altaba and Jessell, 1992). During gastrulation (stage 11-13), *vhh-1* expression was detected in the prechordal plate and notochord with the exception of the posterior region near the blastopore (Fig. 18D). At these stages, expression of *vhh-1* in the notochord was higher dorsally than ventrally (Fig. 18F, G) in contrast to the uniform expression of *Pintallavis*, *brachyury*, *Xlim-1* and *Xnot* mRNAs (Fig. 18I; Smith et al., 1991; Ruiz i Altaba and Jessell, 1992; Taira et al., 1992; von Dassow et al., 1993). At gastrula stages, *Pintallavis* was also expressed in the prechordal plate (Figs. 18E). By the early neurula stage (~stage 15), the level of *vhh-1* in the notochord decreased markedly (Figs. 18H, J) in parallel with the decrease in *Pintallavis* expression (Ruiz i Altaba and Jessell, 1992). At early neural tube stages (~stages 20-26) there was little or no expression of *vhh-1* in the notochord, but expression in the prechordal plate was maintained at high levels until tailbud stages (Figs. 18K, L). At tadpole stages, *vhh-1* was reexpressed transiently in the notochord (stage ~36; Figs. 18M, N), when low levels of HNF-3 $\beta$  are detected (Fig. 18O; Roelink et al., 1994 and not shown).

Neural expression of *vhh-1* was first detected along the entire anteroposterior (A-P), later rostrocaudal, axis (Fig. 18J) in median deep (md) but not median superficial (ms) cells (Schroeder, 1970, ~stage 12-15, Fig. 18G, H). The onset of *vhh-1* expression occurred after that of *Pintallavis* (compare Figs. 18F, G and I). From the early tailbud stage (stage ~24) onwards, however, *vhh-1* was expressed in all floor plate cells at the ventral midline of the midbrain, hindbrain and spinal cord (stage ~36, Fig. 18M, N). Expression of *vhh-1* in the floor plate

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persisted at high levels up to stage ~51, the latest stage examined (not shown). At tadpole stages, floor plate cells expressed both *vhh-1* and *HNF-3 $\beta$*  (Fig. 18M-P). However, unlike *HNF-3 $\beta$*  (Figure 18P; see also Ruiz i Altaba et al., 1993b), *vhh-1* was not expressed in ventricular zone cells immediately adjacent to the floor plate (Fig. 18N).

In the prospective forebrain, expression of *vhh-1* was first detected at neurula stages (~stage 15) initially at the ventral midline of the diencephalon (Fig. 18J and not shown). At tailbud stages, *vhh-1* was expressed throughout the ventral diencephalon (Fig. 18K) extending more dorsally in caudal regions (unlabeled arrow in Fig. 18L, M) paralleling that of *HNF-3 $\beta$*  (unlabeled arrow in Fig. 18O; Ruiz i Altaba et al., 1993b). By the late tailbud to tadpole stages (stages ~28-41) expression of *vhh-1* in the mid-diencephalon was no longer detected at the ventral midline, and instead occupied a more dorsal position (Fig. 18L, M and not shown). In the most rostral diencephalon, the ventral midline expression of *vhh-1* was maintained (Figs. 18M) and a new site of expression of *vhh-1* was detected in ventral telencephalic cells, beginning at stage ~41 (not shown).

*vhh-1* was also expressed in the anterior and posterior endoderm, hypochord, olfactory placode, ventral cells posterior to the heart (Fig. 18L, M and not shown) and in the posterior mesenchyme of the limb buds (not shown), consistent with the pattern of expression of *vhh-1/shh* in other species (Riddle et al., 1993; Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994).

#### **Lack of Neural Expression of *vhh-1* in Exogastrulae**

The expression of *vhh-1* by the floor plate (Fig. 18H, N)



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suggested that *vhh-1* expression in midline cells depends on induction by the notochord. To examine this, complete exogastrula embryos, in which the notochord develops at a distance from the neural ectoderm, were assayed for *vhh-1* expression. In complete exogastrulae (stages ~15 and ~36), *vhh-1* was detected in the notochord and anterior endodermal cells, but not in neural ectoderm (Fig. 18Q and not shown). *Vhh-1* expression by midline neural cells, therefore, appears to depend on signals from the axial mesoderm, consistent with the dependency of *Pintallavis* and HNF-3 $\beta$  expression in floor plate cells on signals from the notochord (Ruiz i Altaba and Jessell, 1992; Dirksen and Jamrich, 1992; Ruiz i Altaba et al., 1993a, 1993b).

#### Localized Plasmid Injections Target Gene Expression to Neural Cells

To examine the effects of *vhh-1* and HNF-3 $\beta$  expression on neural cell patterning, applicants first attempted to establish an injection protocol that would consistently achieve ectopic gene expression in prospective neural cells. The *vhh-1* and HNF-3 $\beta$  genes were inserted into plasmids under the control of a CMV promoter and injected into different regions of frog embryos at the one or two cell stage (Table 6).

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Table 6 - Localization of ectopic HNF-3 $\beta$  neural plate stages (stage approximately 15) after targeted injection of plasmids driving the expression of HNF-3 $\beta$

5		Ectoderm	Neural	Mesoderm
	Injected Region			
10	Equatorial	83%	45%	90%
	Animal	80%	33%	20% <sup>1</sup>
15	Animal pole	90%	70%	19% <sup>2</sup>

Table 6 - Cont'd

20		(Axial)	(Paraxial)	n
	Injected Region			
25	Equatorial	13%	66%	24
	Animal	7%	13%	61
30	Animal pole	n.d.	n.d.	36

Numbers represent percentage of the total number of embryos (n). Expression in ectoderm includes expression in neural tissue. Percentage of embryos showing expression in axial and paraxial mesoderm, but not in more ventral mesoderm, are shown. This value was not determined for injections into the animal pole under the cellular membrane (see text) since only single scattered cells were detected in mesoderm per embryo. Expression of HNF-3 $\beta$  from injected plasmids was driven by a CMV promoter (see Materials and Methods).

1: Large patches of expression in all embryos examined.

2: Only scattered single cells detected in mesoderm.

nd: not determined

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To direct ectopic expression of genes to the neural ectoderm, recombinant plasmids were injected into the extreme animal pole of one or two cell embryos, under the cellular membrane. At gastrula and neural plate stages, ectopic expression of *vhh-1* and HNF-3 $\beta$  was mosaic and detected in large patches in both neural and non-neural ectoderm (Fig. 19A, B, D, E; Tables 6, 7). Targeting of plasmids to the animal pole resulted in expression of the injected genes, predominantly in anterior regions of the embryo (Fig. 19C and not shown). As expected for plasmid injections, ectopic expression of *vhh-1* and HNF-3 $\beta$  was highly mosaic (Fig. 19C, F). Analysis of over 100 injected embryos showed that cells that expressed *vhh-1* or HNF-3 $\beta$  could be found at tadpole stages at any position along the D-V axis of the neural tube (Table 8, Figure 24 and not shown). Thus, injection under the cellular membrane of the animal pole is effective in achieving the expression of genes in the neural ectoderm of frog embryos. Moreover, although the expression of injected *vhh-1* and HNF-3 $\beta$  is mosaic there is no consistent spatial restriction within the neural tube. In these experiments, applicants have assayed mRNA and not protein, and it remains to be established that all cells that express *vhh-1* mRNA can express functional protein.

To determine the effects of misexpression of *vhh-1* and HNF-3 $\beta$  on floor plate differentiation, applicants monitored the expression of four floor plate markers that exhibit distinct temporal patterns of expression. *Pintallavis* is expressed transiently at neural plate stages (Fig. 18; Ruiz i Altaba and Jessell, 1992; Dirksen and Jamrich, 1992) whereas, *vhh-1* is expressed continually from neural plate stages (Fig. 18). *F-spondin*, a gene encoding a floor plate adhesion molecule

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(Klar et al., 1992), and HNF-3 $\beta$  are expressed only after neural tube closure (Fig. 18, Ruiz i Altaba et al., 1993a; Ruiz i Altaba et al., 1993b). Since HNF3 $\beta$  expression appears sufficient to confer floor plate properties to neural tube cells (Sasaki and Hogan, 1994), the combined use of HNF3 $\beta$  with other markers provides a strong case that the induced cells possess floor plate properties. With these markers applicants have examined the timing of ectopic floor plate differentiation and the position at which ectopic floor plate cells appear.

#### Temporal and Spatial Constraints on Floor Plate Induction by *vhh-1*

##### ***vhh-1* Does not Induce the Ectopic Expression of Floor Plate Markers at Neural Plate Stages**

After injection of a plasmid expressing frog or rat *vhh-1*, large patches of cells expressing *vhh-1* were detected in the ectoderm at late blastula/early gastrula stages and in the neural plate at neurula stages (Fig. 19A, B and not shown). At neural plate stages, however, ectopic expression of *Pintallavis* was not detected in the neural ectoderm (Table 7) even though at this time endogenous *Pintallavis* expression occurs in cells at the midline of the neural plate (Ruiz i Altaba and Jessell, 1992; Fig. 18E, I). Similarly, injection of frog *vhh-1* plasmids did not induce the expression of HNF-3 $\beta$  at neural plate stages (Table 7).

Table 7 - Summary of the incidence of ectopic expression of floor plate markers in injected embryos

5	Neural Plate			
	<u>Pintallavis</u>	<u>vhh-1</u>	<u>HNF-3<math>\beta</math></u>	
10	Injected Plasmid			
	<u>vhh-1 s</u>	0/108	12/14	0/42
15	<u>vhh-1 a</u>	0/93	n.d. <sup>1</sup>	n.d.
	<u>R vhh-1 s</u>	0/53	5/21 <sup>3</sup>	n.d.
	<u>R vhh-1 a</u>	0/147	0/72	n.d.
20	<u>HNF-3<math>\beta</math></u>	0/85	0/59	32/36
	<u>HNF-3<math>\beta</math><sup>a</sup></u>	0/43	0/62	+ <sup>6</sup>
25	<u>Table 7 - Cont'd</u>			
30	Neural Tube			
	<u>vhh-1</u>	<u>HNF-3<math>\beta</math></u>	<u>F-spondin</u>	
	Injected Plasmid			
35	<u>vhh-1 s</u>	n.d.	27/164 <sup>2</sup>	n.d.
	<u>vhh-1 a</u>	n.d.	0/108	n.d.
	<u>R vhh-1 s</u>	23/128 <sup>4</sup>	19/153 <sup>5</sup>	22/179 <sup>5</sup>
40	<u>R vhh-1 a</u>	3/112	0/57 <sup>5</sup>	4/198 <sup>5</sup>
	<u>HNF-3<math>\beta</math></u>	80/134	49/61	8/40
45	<u>HNF-3<math>\beta</math><sub>A</sub></u>	5/122	+ <sup>6</sup>	0/55

50 Fractions refer to the number of embryos showing ectopic expression as a function of the total number of embryos assayed. Injected embryos were assayed at neural plate (stages 14-16) or neural tube stages (stages 28-38). The markers assayed in each case are shown on top of each

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- column. The injected genes, cloned in CMV plasmids, are shown at the left of each row. See text for other details. s = sense construct, a: antisense construct, *HNF-3 $\beta$  $\Delta$*  = denotes a truncated *HNF-3 $\beta$*  gene (see Experimental Methods). The few ectopic sites of *vhh-1* and *HNF-3 $\beta$*  expression detected in embryos injected with CMV plasmids driving the expression of antisense *vhh-1* or *HNF-3 $\beta$  $\Delta$*  are detected in dorsal regions. The majority of affected embryos displayed more than 1 site of ectopic floor plate marker expression.
- 1: 40/48 embryos expressed the injected antisense *vhh-1* plasmid.
  - 2: 27/164 embryos expressed ectopic *HNF-3 $\beta$*  in the neural tube. An additional 40/164 embryos expressed ectopic *HNF-3 $\beta$*  exclusively in the otic vesicle. Expression in cells located between the dorsal hindbrain and the otic vesicle was detected rarely (2/16 embryos). Within the neural tube there was only one ectopic site in the telencephalon.
  - 3: Only scattered single cells in the neural plate and adjacent ectoderm (see text).
  - 4: 23/128 embryos expressed *vhh-1* both in the ectoderm and neural tube. An additional 61/128 embryos expressed ectopic *vhh-1* in non-neuronal ectoderm exclusively.
  - 5: Data from Roelink et al. (1994). Injected rat *vhh-1* expression was detected in 11/11 embryos at neural plate stages and in 23/74 embryos at tadpole stages.
  - 6: *HNF-3 $\beta$*  protein is detected in the nucleus. *HNF-3 $\beta$  $\Delta$*  protein is detected both in the cytoplasm and nucleus.
  - nd: not determined.

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Since *vhh-1* is expressed by cells at the midline of the neural plate (Figs. 18G, H), applicants tested whether *vhh-1* could induce its own expression by injecting rat *vhh-1* plasmids and assaying for the expression of frog *vhh-1*. In the vast majority of embryos no ectopic expression of *vhh-1* was apparent, but in a few embryos, scattered cells that expressed *vhh-1* were detected in the neural plate and in the adjacent ectoderm (Fig. 21A; Table 7).

These results provide evidence that floor plate genes are not induced ectopically at neural plate stages in response to widespread expression of *vhh-1*.

#### **Ectopic induction of Floor Plate Markers occurs at Neural Tube Stages in Response to *vhh-1***

Expression of floor plate markers was detected ectopically in injected embryos that developed to neural tube stages. Ectopic expression of HNF-3 $\beta$  was detected after injection of frog (Fig. 20; Table 7) or rat *vhh-1* plasmids (Roelink et al., 1994; Table 7). Injection of plasmid constructs driving the expression of *vhh-1* in the antisense orientation did not lead to the ectopic expression of HNF-3 $\beta$  (Table 7). Injection of rat *vhh-1* also resulted in the ectopic expression of frog *vhh-1* within the neural tube (Fig. 21, Table 7) and in the non-neural ectoderm (Table 7). Injection of an antisense rat *vhh-1* plasmid resulted in only a very low incidence of ectopic expression of frog *vhh-1* mRNA (Table 7). Previous studies have shown that widespread expression of rat *vhh-1* also leads to the ectopic expression of F-spondin (Roelink et al., 1994).

The ectopic dorsal expression of *vhh-1* and HNF-3 $\beta$  was observed in the spinal cord, hindbrain, midbrain and

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diencephalon but only rarely in the telencephalon (data not shown). The low incidence of ectopic floor plate marker expression in the telencephalon is striking since anterior regions of the embryo displayed a high incidence of expression of injected plasmids (Fig. 19B, C).

Taken together, these results indicate that widespread expression of *vhh-1* leads to the ectopic differentiation of floor plate cells within the neural tube.

#### **Ectopic Floor Plate Differentiation Induced by *vhh-1* is Restricted**

Although both HNF-3 $\beta$  and *vhh-1* are expressed ectopically in the neural tube of injected embryos there were marked spatial restrictions in the pattern of ectopic gene expression. Analysis by whole-mount showed that all affected embryos exhibited dorsal sites of ectopic gene expression (Figs. 20, 21). In addition, HNF-3 $\beta$  and *vhh-1* expression occasionally occupied the D-V extent of the neural tube (23% of *vhh-1* sites, n=35 sites; see Fig. 21B and 10% of HNF-3 $\beta$  sites, n=40 sites; not shown). In a lower proportion of sites, ectopic floor plate marker expression appeared as an expansion of the normal ventral midline domain of expression of floor plate genes (9% of *vhh-1* sites, not shown and 10% of HNF-3 $\beta$  sites; see Fig. 20B).

To determine more precisely the sites of ectopic floor plate marker expression, transverse sections of the neural tube of injected embryos were examined (Table 8 and Figure 24). The majority of ectopic sites were found in and around the roof plate (Figs. 20A-E; 20B-D, F). Cells in the most dorsal region of the alar plate immediately adjacent to the roof plate also expressed floor plate markers at a lower incidence (arrow in Fig.



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20D). In more ventral regions of the neural tube, ectopic floor plate markers were often expressed along the ventricular zone (Table 8 and Figure 24). Ectopic floor plate marker expression was not detected in lateral regions of the alar of basal plates (Figs. 20D-F, 21D, F; Table 8 and Figure 24). Embryos in which ectopic expression of *vhh-1* or HNF-3 $\beta$  were detected often exhibited changes in neural tube morphology, most frequently a branched neural tube (Figs. 20E, 21E, 21F).

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Table 8 - Localization of ectopic sites of floor plate marker expression within the neural tube of injected embryos

5		Injected Plasmid	Marker	RP	DAP	AP/BP	VZ	V
10	Rvhh-1	vhh-1	71	18	0	29	6	
	vhh-1	HNF-3 $\beta$	74	26	0	9	11	
	HNF-3 $\beta$	vhh-1	81	0	0	23	4	
15	HNF-3 $\beta$	HNF-3 $\beta$	47	3	87	0	0	
	Percentage of Cells			7	8	57	22	4

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Table 8 - Cont'd

25	Injected Plasmid	Marker	FP	n
	Rvhh-1	vhh-1	+	17
	vhh-1	HNF-3 $\beta$	+	35
30	HNF-3 $\beta$	vhh-1	+	26
	HNF-3 $\beta$	HNF-3 $\beta$	+	30
35	Percentage of Cells		2	171

40 Numbers refer to percentage of cases in each zone (see Figure 24) as a function of the total number of cases (n). Some sites of expression spanned two or more zones. Each row shows the results of expression of the specified marker (top right columns), *vhh-1* mRNA or HNF-3 $\beta$  protein, after injection of CMV plasmids driving the expression of rat *vhh-1* (Rvhh-1), frog *vhh-1* or frog HNF-3 $\beta$  (left of each row). The localization of ectopic *F-spondin* sites is not shown since only a small number of sites were analyzed. Number of cells (bottom row) represent the average percentage of cells located within each zone unilaterally. Average were determined counting the numbers of DAPI stained nuclei in one half of 3 different sections. Numbers were obtained by inspection of transverse sections.

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### Temporal and Spatial Constraints on Floor Plate Induction by HNF-3 $\beta$

The temporal and spatial restrictions in floor plate induction observed after widespread expression of *vhh-1* described above, could in principle occur upstream of, or in parallel with the induction of *Pintallavis* and HNF-3 $\beta$  expression. If such restrictions occur upstream of *Pintallavis* or HNF-3 $\beta$  activation, they might not be evident in response to widespread expression of HNF-3 $\beta$ . Applicants therefore assessed possible restrictions in floor plate induction by HNF-3 $\beta$ .

### HNF-3 $\beta$ does not Induce the Ectopic Expression of Floor Plate Markers at Neural Plate Stages

Ectopic expression of *Pintallavis* or *vhh-1* was not detected in the neural plate of embryos injected with HNF-3 $\beta$  plasmids (Table 7). The temporal restriction in floor plate marker expression observed in response to *vhh-1* are, therefore, also evident after widespread expression of HNF-3 $\beta$ .

### Ectopic Induction of Floor Plate Markers Occurs at Neural Tube Stages in Response to HNF-3 $\beta$

Ectopic expression of *vhh-1* and F-spondin was detected in the neural tube in a high proportion of embryos that expressed injected HNF-3 $\beta$  (Fig. 22A, B, D, F; Table 7). Injection of plasmids driving the expression of a truncated HNF-3 $\beta$  gene (see Experimental Methods) did not result in ectopic expression of *vhh-1* or F-spondin (Table 7). These results are consistent with previous studies showing that widespread expression of *Pintallavis* induces the ectopic expression of F-spondin at tadpole stages (Ruiz i Altaba et al., 1993a). Widespread expression of HNF-3 $\beta$  was able to induce ectopic floor plate marker expression along the A-P axis of the neural tube (Fig.

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22A). In the telencephalon however, only a single ectopic site was found. Thus, HNF-3 $\beta$  can induce the ectopic expression of *vhh-1* and other floor plate markers within the neural tube.

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**Ectopic Floor Plate Differentiation Induced by HNF-3 $\beta$  is Spatially Restricted**

The ectopic expression of both *vhh-1* or *F-spondin* detected after widespread expression of HNF-3 $\beta$  showed marked restrictions within the neural tube. Wholemout analysis showed that widespread expression of HNF-3 $\beta$  resulted in the preferential localization of ectopic floor plate markers to the dorsal neural tube (Fig. 22; Table 8 and Figure 24) with all affected embryos showing dorsal ectopic expression sites. In addition, at 23% of sites, *vhh-1* expression spanned the D-V extent of the neural tube and at 8% of sites *vhh-1* was expressed in an expanded ventral region (n=60 sites; not shown; see also Ruiz i Altaba et al., 1993a).

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Examination of transverse sections revealed that most of the ectopic *vhh-1* sites were found dorsally (Table 8 and Figure 24). In more ventral regions of the neural tube, ectopic *vhh-1* expression was restricted either to the ventricular zone, often unilaterally, or to cells immediately adjacent to the floor plate, usually in the ventricular zone (Table 8 and Figure 24). Ectopic *vhh-1* or *F-spondin* expression was not detected in lateral regions of the alar or basal plates (Fig. 22D, F; Table 8 and Figure 24 and not shown). Neural tube malformations were often accompanied by ectopic *vhh-1* expression (not shown).

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These results demonstrate that HNF-3 $\beta$  can activate the transcription of *vhh-1* and other floor plate markers in

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neural tube cells and that the spatial restrictions in floor plate marker expression detected in response to *vhh-1* are also evident after widespread expression of HNF-3 $\beta$ .

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#### EXPERIMENTAL DISCUSSION

##### **Reciprocal Activation of *vhh-1* and Winged-Helix Genes and the Homeogenetic Nature of Floor Plate Induction**

10 The differentiation of floor plate cells at the midline of the neural plate is induced by signals from the notochord (van Straaten et al., 1988; Placzek et al., 1990, 1993; Hatta, 1991; Yamada et al., 1991; Ruiz i Altaba, 1992; Jessell and Dodd, 1992). Once induced,  
15 floor plate cells acquire the ability to induce the differentiation of additional floor plate cells (Placzek et al., 1990; 1993; Yamada et al., 1991; Hatta et al., 1991). Thus, induction of floor plate differentiation is a homeogenetic process in which cells of the notochord  
20 confer similar signalling properties to midline neural plate cells. The present studies on *vhh-1* and HNF-3 $\beta$ , taken together with previous findings (Ruiz i Altaba et al., 1993a; Sasaki and Hogan, 1994; Krauss et al., 1993; Echelard et al., 1993; Roelink et al., 1994) suggest a  
25 molecular pathway for floor plate induction and mechanisms that could underly the propagation and eventual restriction of this inductive process (Fig. 23).

*Pintallavis* is expressed in the organizer region and the  
30 notochord prior to the onset of *vhh-1* expression. In frog embryos *Pintallavis* appears to assume the early functions ascribed to HNF-3 $\beta$  in the mouse (Ruiz i Altaba et al., 1993b) and thus may be required for the expression of *vhh-1* in the notochord. It remains  
35 unclear, however, whether *vhh-1* represents a direct

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target of winged-helix transcription factors. *vhh-1* expression in the notochord precedes that of floor plate markers in cells at the midline of the neural plate (Fig. 18; Ruiz i Altaba and Jessell, 1992) and *vhh-1* can induce ectopic expression of floor plate markers (Figs. 20, 21; Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994). Thus, it is likely *vhh-1/shh* secreted by the notochord participates normally in the induction of floor plate differentiation.

Three lines of evidence indicate that the induction of *Pintallavis* and HNF-3 $\beta$  in midline neural cells is required for floor plate differentiation. First, the expression of *Pintallavis* in frog and HNF-3 $\beta$  in chicks appear to be direct responses of neural plate cells to notochord-derived inductive signals (Ruiz i Altaba et al., 1993a; 1995). Second, both *Pintallavis* and HNF-3 $\beta$  can induce the ectopic expression of floor plate markers in the neural tube (Fig. 22; Ruiz i Altaba et al., 1993a, Sasaki and Hogan, 1994) including *vhh-1/shh* (Fig. 22). Third, separating the notochord from the ectoderm leads to the lack of expression of *Pintallavis* and HNF-3 $\beta$  and other floor plate markers in the neural ectoderm (fig. 1Q; Ruiz i Altaba, 1994). The floor plate attains autonomy from the notochord around the time of neural tube closure (Yamada et al., 1991; Placzek et al., 1991). Such autonomy may be established by the autoregulation of HNF-3 $\beta$  which has been shown to occur in vitro (Pani et al., 1992) and in the neural tube in vivo (Fig. 19F, 22C; Sasaki and Hogan, 1994).

Taken together, these experimental observations are consistent with a model in which the sequential expression of winged-helix transcription factors and vertebrate *hedgehog* genes by the notochord underlies the

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initial phase of floor plate induction. The sequential expression of these genes in the floor plate may also participate in the homeogenetic induction of additional floor plate cells. In vivo, however, this signalling cascade is not propagated indefinitely throughout the neural plate and neural tube. The extent of floor plate differentiation may be limited in part by the range of action of secreted *vhh-1* and, as discussed below, by restrictions in the ability of neural cells to respond to *vhh-1* and winged-helix factors.

#### Constraints on Ectopic Floor Plate Induction

The main finding of the present work is that there are marked temporal and spatial constraints on the ability of *vhh-1* and winged-helix transcription factors to induce floor plate differentiation.

During normal development, floor plate markers are first expressed by cells at the midline of the neural plate (Figs. 18, 23). In contrast, misexpression of *vhh-1* or HNF-3 $\beta$  fails to induce ectopic expression of floor plate markers in neural plate cells (Fig. 24). It is unlikely that lateral neural plate cells express *vhh-1* or HNF-3 $\beta$  and then die since these cells can express the same genes driven by a plasmid vector (Table 8, Figure 24 and not shown). One possible explanation for the observed restrictions in floor plate differentiation is that the notochord provided two signals, a vertebrate hedgehog protein and a distinct factor, with the combined action of both signals being required to trigger floor plate differentiation at neural plate stages. A second possibility is that the inability of lateral neural plate cells to respond to *vhh-1* and HNF-3 $\beta$  is imposed by signals derived from non-neural tissues, in particular, from paraxial mesoderm that underlies the lateral region

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of the neural ectoderm. The only neural plate cells capable of responding to *vhh-1* and HNF-3 $\beta$  would, therefore, be those at the midline which are removed from a local inhibitory influence of paraxial mesoderm by virtue of their apposition with the notochord. In either case, these temporal restrictions in floor plate differentiation are observed when the extopic expression of HNF-3 $\beta$  is induced by *vhh-1* and when the expression of *vhh-1* is induced by HNF-3 $\beta$ . Thus, these restrictions appear to act both upstream and downstream of HNF-3 $\beta$ .

After neural tube closure, neural cells can respond to widespread expression of *vhh-1* and HNF-3 $\beta$  with ectopic floor plate differentiation. Ectopic floor plate cells are, however, confined primarily to the dorsal neural tube and to cells in the ventricular zone (Fig. 24). The constraints that operate at neural plate stages might, therefore, be maintained after neural tube closure with the exception of cells in the most dorsal region of the neural tube stages and in the ventricular zone. An additional constraint that could contribute to the spatial restrictions on ectopic floor plate differentiation at neural tube stages is neuronal differentiation. The exclusion of floor plate gene expression from neurons might confine ectopic floor plate differentiation primarily to ventricular zone cells and to the non-neural cells of the roof plate.

The absence of ectopic floor plate differentiation in intermediate regions of the neural tube of frog embryos contrasts with the ability of a secondary notochord to induce a floor plate in this region of the chick and frog neural tube (Yamada et al., 1991; ARA and TMJ, unpublished) and with the ability of *vhh-1* expressed in COS cells to induce floor plate differentiation in rat



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lateral neural plate explants in vitro (Roelink et al., 1994). These differences could be explained by the action in vivo of a repressive signal that derives from paraxial mesoderm. Notochord grafts physically separate the neural plate from the somites, removing neural plate cells from the local influence of such a signal. Similarly, isolation of neural plate explants in vitro removes neural cells from signals derived from surrounding tissues and thus may permit floor plate differentiation in response to *vhh-1*.

#### **Contribution of Spatial Restrictions to Normal Floor Plate Differentiation**

Floor plate cells differentiate in a restricted domain at the ventral midline of the neural tube (Fig. 23). The initial induction of floor plate differentiation by the notochord appear to be mediated by a contact-dependent signal (Placzek et al., 1993). Thus, the spatial restriction in floor plate differentiation could depend on the limited extent of contact between the notochord and neural plate cells. However, induced floor plate cells acquire the capacity to induce new floor plate cells through homeogenetic induction (Hatta et al., 1991; Yamada et al., 1991; Placzek et al., 1993). Restriction on the spread of floor plate differentiation, therefore, appear to operate during normal development.

In vivo and in vitro studies have shown that neural cells have a limited period of competence to respond to floor plate inducing signals (van Straaten et al., 1988; Yamada et al., 1991; Placzek et al., 1993). Thus, the spread of floor plate induction may be limited, in part, by the loss of competence of neural cells to respond to inductive signals. Applicants' in vivo studies show, however, that the widespread expression of *vhh-1* or HNF-

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3 $\beta$  cannot drive the ectopic expression of floor plate markers in the neural plate. In vivo, therefore, there be constraints on the propagation of floor plate differentiation that act prior to and independent of the  
5 loss of competence of neural cells (Fig. 23).

#### ***vhh-1*, Winged-Helix Genes and Forebrain Patterning**

In the neural tube, the expression *vhh-1* includes floor plate cells and midline cells of the forebrain. One  
10 possible source of inductive signals responsible for *vhh-1* expression in the rostral forebrain is the prechordal plate, which has been implicated in the progression of forebrain differentiation (Dixon and Kintner, 1989; Ruiz i Altaba, 1992). Both *Pintallavis* and *vhh-1* are  
15 expressed in the prechordal plate. Thus, expression of *vhh-1* in the prechordal plate mesoderm might be regulated by winged-helix transcription factors in a manner similar to that occurring in the notochord. In view of the participation of notochord-derived *vhh-1* in the induction  
20 of floor plate properties at posterior levels of the neuraxis, it is also possible that *vhh-1* secreted by the prechordal plate is involved in the induction of *vhh-1* in midline cells of the rostral forebrain. However, neither *Pintallavis*, HNF-3 $\beta$  nor HNF-3 $\alpha$  are expressed in the  
25 rostral forebrain at the time when *vhh-1* mRNA first appears. Thus, *vhh-1* expression this region is likely to be regulated by a pathway distinct from that operating to induce *vhh-1* expression in floor plate cells.

#### **Experimental Methods**

##### **Frogs, Embryos and Microinjection**

*Xenopus laevis* female frogs were induced to lay eggs by injection of 1000 u. of human chorionic gonadotropin.  
35 Eggs were fertilized with testis homogenates and reared

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under standard conditions (Ruiz i Altaba, 1993). Staging of embryos was according to Nieuwkoop and Faber (1967).

5 Fertilized eggs were dejellied in 3% cysteine pH 7.6 before first cleavage and transferred to injection solution (3% ficoll, 1xMMR). Injection was performed as described (Ruiz i Altaba, 1993) before or after first cleavage. In the majority of cases injection was targeted to the animal pole (see text). Because the  
10 formation of the first cleavage furrow begins in this area, embryos frequently received an injection into a single blastomere which resulted in the unilateral distribution of injected materials. Injected embryos were cultured in injection solution for about 1 hour and  
15 then transferred gradually to 0.1xMMR.

100-200 pg of supercoiled plasmid DNA in water was injected into frog embryos and was not detrimental for embryonic development. Large amounts of plasmid DNA were  
20 toxic.

#### Library Screens and Clones

To isolate a frog *vhh-1* cDNA, 10<sup>6</sup> recombinant phages of a *Xenopus laevis* stage 17 whole embryo library (Kintner  
25 and Melton, 1987) were screened with the full-length rat *vhh-1* cDNA (Roelink et al., 1994) at moderate stringency in HM: 10% dextran sulphate, 3 x SSC, 3 x SSPE, 5 x Denhardt's, 0.5% SDS and 100 µg/ml denatured herring sperm DNA at 60°C. Nitrocellulose filters were washed in  
30 1xSSC, 0.1% SDS for 2-4 h. Of 50 positive plaques 10 were analysed further. Applicants isolated the two copies of the *vhh-1* gene in the *Xenopus* tetraploid genome and other members of the *hh* gene family.

35 Lambda clone #4 was digested with EcoRI and the ~2.4 Kb

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insert subcloned into pBluescript SK yielding pfhh #4. The nucleotide sequence of this insert was determined on both strands by the chain termination method using dsDNA as template and Sequenase (USB). Sequence analysis was performed with a VAX computer.

For injection, the EcoRI *vhh-1* cDNA insert of pfhh #4 was cloned into pcDNA1-Amp (Invitrogen) which contains a cytomegalovirus (CMV) promoter 5' to the polylinker and SV40 polyadenylation sequences 3' to the polylinker. Two clones were made with *vhh-1* in the sense and antisense orientations and named pCMV-*vhh-1* S and pCMV-*vhh-1* A. Similarly, the EcoRI-Not I HNF-3 $\beta$  cDNA fragment of X $\beta$ 1 (Ruiz i Altaba et al., 1993b) was cloned into pcDNA1-Amp yielding pCMV-X $\beta$ . As control, pCMV-X $\beta$  was cut at the single BglII site, filled-in and religated yielding pCMV-X $\beta$  $\Delta$ . This mutation changes the reading frame downstream of the BglII site adding 30 amino acids before terminating prematurely. The X $\beta$  $\Delta$  protein product lacks most of the DNA-binding domain conserving only helix 1 and two amino acids of helix 2 (see Clark et al., 1993 and Ruiz i Altaba et al., 1993b). The X $\beta$  $\Delta$  protein is predicted to lack DNA-binding activity.

#### In Situ Hybridization

Frog embryos were processed for whole-mount in situ hybridization as described by Harland (1991). The vitelline membrane of young embryos was removed manually and holes were made into the blastocoel and archenteron to prevent background labelling. Embryos were fixed in MEMFA (3.7% formaldehyde, 1 mM EGTA, 2 mM MgCl<sub>2</sub>, 0.1M MOPS; Patel et al., 1989) for 2 h, dehydrated and stored in 100% methanol at -20°C. Embryos were not prehybridized and the RNA probes were not hydrolyzed. Detection of specific hybridization was performed with an anti-

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digoxigenin antibody coupled to alkaline phosphatase and reacted with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate.

5 Single-stranded digoxigenin-labelled antisense and sense RNA probes were generated by in vitro transcription of the appropriate plasmid clones in the presence of digoxigenin-UTP and a trace of <sup>32</sup>P-UTP to measure incorporation. An antisense frog *vhh-1* RNA probe  
10 spanning the entire cDNA clone was generated by transcribing NotI cut pfhh#4 with T3 RNA polymerase. An identical pattern of *vhh-1* expression was observed with an antisense probe spanning only the 3' untranslated region. A sense frog *vhh-1* RNA probe was generated by  
15 transcribing SalI cut pfhh#4 with T7 RNA polymerase. An antisense rat *vhh-1* RNA probe was generated by transcribing Bam HI cut pRvhh-1#7 (Roelink et al., 1994) with T3 RNA polymerase. Hybridization of embryos at different stages with the rat *vhh-1* antisense probe did  
20 not reveal the pattern of expression of frog *vhh-1* mRNA showing that the frog and rat probes do not cross-hybridize. An antisense *Pintallavis* RNA probe was generated by transcribing HindIII cut pF5 (Ruiz i Altaba and Jessell, 1992) with T7 RNA polymerase. An antisense  
25 *gooseoid* RNA probe was generated by transcribing an EcoRI cut 0.9 Kb PCR clone derived from stage 10 dorsal lip cDNA with T7 RNA polymerase.

#### Immunocytochemistry

30 Whole-mount antibody labelling was performed as described by Dent et al. (1989) and Patel et al. (1989). Embryos were fixed for ~20 min. in MEMFA and bleached in 10% H<sub>2</sub>O<sub>2</sub> in methanol overnight under fluorescent light at 4°C. Embryos were gradually transferred to PBS, washed  
35 extensively in PBS plus 0.1% Triton X-100 (PBT) and

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blocked in PBT plus 10% heat-inactivated goat serum at room temperature for ~1h. Primary antibody incubation was carried out at 4°C overnight on a nutator (Adams). After four to five 30 min. washes in PBT at room temperature, embryos were incubated with goat anti-rabbit secondary antibodies coupled to horseradish peroxidase (1/100; Boehringer Mannheim) and reacted for 2 h. at room temperature on a nutator. Embryos were then washed at least five times, for a total of 2-3h, and reacted with  $H_2O_2$  in the presence of diaminobenzidine. Embryos were dehydrated and cleared in benzyl alcohol/benzy benzoate (1/2) before viewing with an axiophot (Zeiss) microscope under Nomarski optics.

15 Rabbit anti-HNF-3 $\beta$  antibodies were generated by immunizing female New Zealand white rabbits with a 30 amino acid peptide corresponding to the amino terminal end of the frog HNF-3 $\beta$  protein (Ruiz i Altaba et al., 1993b) containing a C-terminal cysteine coupled to

20 activated keyhole limpet hemocyanin (Pierce).

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## SEQUENCE LISTING

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Roelink, Henk  
Edlund, Thomas
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THEREOF
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30 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
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(B) FILING DATE:  
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50 (A) LENGTH: 1715 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- 55 (ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 315..1628
- 60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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	Arg Gly Phe Gly Lys Arg Gln His Pro Lys Lys Leu Thr Pro Leu Ala	
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	Tyr Lys Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser	
	45 50 55 60	
	GGC CGA TAT GAA GGG AAG ATC ACA AGA AAC TCC GAA CGA TTT AAG GAA	542
	Gly Arg Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu	
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35	ACT GGA GCA GAC CGG CTG ATG ACT CAG AGG TGC AAA GAC AAG TTA AAT	638
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	His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg	
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10	AGC GAC TTC CTC ACC TTC CTG GAC CGC GAC GAA GGT GCC AAG AAG GTC	1070
	Ser Asp Phe Leu Thr Phe Leu Asp Arg Asp Glu Gly Ala Lys Lys Val	
	240 245 250	
15	TTC TAC GTG ATC GAG ACG CGG GAG CCG CGG GAG CGT CTG CTG CTC ACT	1118
	Phe Tyr Val Ile Glu Thr Arg Glu Pro Arg Glu Arg Leu Leu Leu Thr	
	255 260 265	
20	GCC GCG CAC CTG CTC TTC GTG GCG CCG CAC AAC GAC TCC GGG CCC ACT	1166
	Ala Ala His Leu Leu Phe Val Ala Pro His Asn Asp Ser Gly Pro Thr	
	270 275 280	
25	CCG GGA CCG AGC CCA CTC TTC GCC AGC CGC GTG CGT CCG GGG CAG CGC	1214
	Pro Gly Pro Ser Pro Leu Phe Ala Ser Arg Val Arg Pro Gly Gln Arg	
	285 290 295 300	
30	GTG TAC GTG GTG GCT GAA CGC GGC GGG GAC CGC CGG CTG CTG CCC GCC	1262
	Val Tyr Val Val Ala Glu Arg Gly Gly Asp Arg Arg Leu Leu Pro Ala	
	305 310 315	
35	GCG GTG CAC AGC GTA ACG CTA CGA GAG GAG GCG GCG GGT GCG TAC GCG	1310
	Ala Val His Ser Val Thr Leu Arg Glu Glu Ala Ala Gly Ala Tyr Ala	
	320 325 330	
40	CCG CTC ACG GCG GAC GGC ACC ATT CTC ATC AAC CGG GTG CTC GCC TCG	1358
	Pro Leu Thr Ala Asp Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser	
	335 340 345	
45	TGC TAC GCA GTC ATC GAG GAG CAC AGC TGG GCA CAC CGG GCC TTC GCG	1406
	Cys Tyr Ala Val Ile Glu Glu His Ser Trp Ala His Arg Ala Phe Ala	
	350 355 360	
50	CCC TTC CGC CTG GCG CAC GCG CTG CTG GCC GCG CTG GCA CCC GCC CGC	1454
	Pro Phe Arg Leu Ala His Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg	
	365 370 375 380	
55	ACG GAC GGC GGG GGC GGG GGC AGC ATC CCT GCC CCG CAA TCT GTA GCG	1502
	Thr Asp Gly Gly Gly Gly Ser Ile Pro Ala Pro Gln Ser Val Ala	
	385 390 395	
60	GAA GCG AGG GGC GCA GGG CCG CCT GCG GGC ATC CAC TGG TAC TCG CAG	1550
	Glu Ala Arg Gly Ala Gly Pro Pro Ala Gly Ile His Trp Tyr Ser Gln	
	400 405 410	
65	CTG CTG TAC CAC ATT GGC ACC TGG CTG TTG GAC AGC GAG ACC CTG CAT	1598
	Leu Leu Tyr His Ile Gly Thr Trp Leu Leu Asp Ser Glu Thr Leu His	
	415 420 425	
70	CCC TTG GGA ATG GCA GTC AAG TCC AGC TGAAGTCCGA CGGGACCGGG	1645
	Pro Leu Gly Met Ala Val Lys Ser Ser	
	430 435	
75	CAGGGGGCGT GGGGGCGGGC GGGGCGGGAA GCGACTGCCA GATAAGCAAC CGGGAAAGCG	1705

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CACGGAAGGA

1715

## (2) INFORMATION FOR SEQ ID NO:2:

5

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 437 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

10

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

15

Met Leu Leu Leu Leu Ala Arg Cys Phe Leu Val Ala Leu Ala Ser Ser  
1 5 10 15Leu Leu Val Cys Pro Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly  
20 25 30

20

Lys Arg Gln His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe  
35 40 45

25

Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu  
50 55 60Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn  
65 70 75 80

30

Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp  
85 90 95Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile  
100 105 110

35

Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly  
115 120 125

40

Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly  
130 135 140Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly  
145 150 155 160

45

Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr  
165 170 175Glu Ser Lys Ala Arg Ile His Cys Ser Val Lys Ala Glu Asn Ser Val  
180 185 190

50

Ala Ala Lys Ser Asp Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu  
195 200 205

55

Glu Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly Asp Arg  
210 215 220Val Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu  
225 230 235 240

60

Thr Phe Leu Asp Arg Asp Glu Gly Ala Lys Lys Val Phe Tyr Val Ile  
245 250 255

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Glu Thr Arg Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu  
                   260                                  265                                  270  
 5   Leu Phe Val Ala Pro His Asn Asp Ser Gly Pro Thr Pro Gly Pro Ser  
                   275                                  280                                  285  
 Pro Leu Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val  
                   290                                  295                                  300  
 10   Ala Glu Arg Gly Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser  
                   305                                  310                                  315                                  320  
 Val Thr Leu Arg Glu Glu Ala Ala Gly Ala Tyr Ala Pro Leu Thr Ala  
                                   325                                  330                                  335  
 15   Asp Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val  
                                   340                                  345                                  350  
 20   Ile Glu Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu  
                   355                                  360                                  365  
 Ala His Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Gly Gly  
                   370                                  375                                  380  
 25   Gly Gly Gly Ser Ile Pro Ala Pro Gln Ser Val Ala Glu Ala Arg Gly  
                   385                                  390                                  395                                  400  
 Ala Gly Pro Pro Ala Gly Ile His Trp Tyr Ser Gln Leu Leu Tyr His  
                                   405                                  410                                  415  
 30   Ile Gly Thr Trp Leu Leu Asp Ser Glu Thr Leu His Pro Leu Gly Met  
                   420                                  425                                  430  
 35   Ala Val Lys Ser Ser  
                   435

## (2) INFORMATION FOR SEQ ID NO:3:

- 40       (i) SEQUENCE CHARACTERISTICS:  
           (A) LENGTH: 20 base pairs  
           (B) TYPE: nucleic acid  
           (C) STRANDEDNESS: single  
           (D) TOPOLOGY: linear  
 45       (ii) MOLECULE TYPE: DNA (genomic)

## 50       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAGGATTGGG TCGTCATAGG

20

## 55       (2) INFORMATION FOR SEQ ID NO:4:

- 60       (i) SEQUENCE CHARACTERISTICS:  
           (A) LENGTH: 20 base pairs  
           (B) TYPE: nucleic acid  
           (C) STRANDEDNESS: single  
           (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTTCCAGGAT TCCATCTCAA

20

10 (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
15 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGCTGGGACG AGGACTACCA TC

22

25

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs  
30 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

40

TGGGAAGTGA TCGACGAATC TG

22

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## What is claimed is:

1. An isolated nucleic acid molecule encoding a vertebrate vhh-1 protein.
- 5 2. An isolated DNA molecule of claim 1.
3. An isolated cDNA molecule of claim 2.
- 10 4. An isolated nucleic acid molecule of claim 1, wherein the nucleic acid molecule encodes a frog vhh-1 protein.
- 15 5. An isolated nucleic acid molecule of claim 1, wherein the nucleic acid molecule encodes a mammalian vhh-1 protein.
- 20 6. An isolated nucleic acid molecule of claim 1, wherein the nucleic acid molecule encodes a rat vhh-1 protein.
- 25 7. An isolated nucleic acid molecule of claim 1, wherein the nucleic acid molecule encodes a human vhh-1 protein.
8. An isolated DNA molecule of claim 4, 5, 6 or 7.
9. An isolated cDNA molecule of claim 8.
- 30 10. A vector comprising the nucleic acid molecule of claim 1.
11. A plasmid comprising the vector of claim 10.
- 35 12. The plasmid of claim 11, designated pMT21 2hh #7

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(ATCC Accession No. 75686).

13. An expression plasmid comprising the nucleic acid molecule of claim 1.
- 5 14. The plasmid of claim 13, which is designated cmv vhh 7 (ATCC Accession No. 75685).
- 10 15. A mammalian cell comprising the plasmid of claim 11 or 13.
16. The mammalian cell of claim 12, wherein the cell is a Cos cell.
- 15 17. A nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule comprising the gene encoding the vertebrate vhh-1 protein.
- 20 18. The nucleic acid probe of claim 17, wherein the nucleic acid molecule is a DNA molecule.
- 25 19. A purified vertebrate vhh-1 protein.
20. A purified unique polypeptide fragment of the vertebrate vhh-1 protein of claim 19.
- 30 21. A purified frog vhh-1 protein.
22. A purified mammalian vhh-1 protein.
- 35 23. A purified unique polypeptide fragment of the mammalian vhh-1 protein of claim 22.

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24. A purified human vhh-1 protein.
25. A monoclonal antibody directed to a vertebrate vhh-1 protein.
- 5 26. A monoclonal antibody of claim 25 directed to a frog vhh-1 protein.
- 10 27. A monoclonal antibody of claim 25 directed to a mammalian vhh-1 protein.
28. A monoclonal antibody of claim 25 directed to a rat vhh-1 protein.
- 15 29. A monoclonal antibody of claim 25 directed to a human vhh-1 protein.
30. Polyclonal antibodies directed to a vertebrate vhh-1 protein.
- 20 31. A transgenic nonhuman mammal which comprises an isolated DNA molecule of claim 2.
- 25 32. The transgenic nonhuman mammal of claim 31, wherein the DNA encoding a vertebrate vhh-1 protein is operatively linked to a tissue specific regulatory elements.
- 30 33. A method of determining the physiological effects of expressing varying levels of vertebrate vhh-1 protein which comprises producing a panel of transgenic nonhuman animals each expressing a different amount of vertebrate vhh-1 protein.
- 35 34. A method of producing the isolated protein of claim

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19 which comprises:

- a. inserting nucleic acid molecule encoding the vertebrate vhh-1 protein in a suitable vector;
  - b. introducing the resulting vector in a suitable host cell;
  - c. selecting the introduced host cell for the expression of the vertebrate vhh-1 protein;
  - d. culturing the selected cell to produce the vhh-1 protein; and
  - e. recovering the vhh-1 protein produced.
35. A method of inducing the differentiation of floor plate cells comprising contacting floor plate cells with the purified vertebrate vhh-1 protein of claim 19 at a concentration effective to induce the differentiation of floor plate cells.
36. A method of inducing the differentiation of floor plate cells in a subject comprising administering to the subject the purified vertebrate vhh-1 protein of claim 19 at an amount effective to induce the differentiation of floor plate cells in the subject.
37. A method of inducing the differentiation of motor neuron comprising contacting the floor plate cells with the purified vertebrate vhh-1 protein of claim 19 at a concentration effective to induce the differentiation of motor neuron.
38. A method of inducing the differentiation of motor



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neuron in a subject comprising administering to the subject the purified vertebrate vhh-1 protein of claim 19 at an amount effective to induce the differentiation of motor neuron in the subject.

5

39. A method of generating ventral neurons comprising contacting progenitor cells with the purified vertebrate vhh-1 protein of claim 19 at a concentration effective to generate ventral neurons.

10

40. A method of generating ventral neurons from progenitor cells in a subject comprising administering to the subject the purified vertebrate vhh-1 protein of claim 19 at an amount effective to generate ventral neurons from progenitor cells in the subject.

15

41. A pharmaceutical composition comprising a purified vertebrate vhh-1 protein of claim 19 and a pharmaceutically acceptable carrier.

20

42. A pharmaceutical composition comprising a purified mammalian vhh-1 protein of claim 22 and a pharmaceutically acceptable carrier.

25

43. A pharmaceutical composition comprising a purified human vhh-1 protein of claim 23 and a pharmaceutically acceptable carrier.

30

44. A pharmaceutical composition comprising a purified human vhh-1 protein of claim 24 and a pharmaceutically acceptable carrier.

35

45. A method for treating a human subject afflicted with an abnormality associated with a lack of one or more

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normally functioning motor neurons which comprises introducing an amount of pharmaceutical composition of claim 41, 42, 43 or 44 effective to generate motor neurons from undifferentiated motor neuron precursor cells in a human, thereby treating a human subject afflicted with an abnormality associated with a lack of one or more normally functioning motor neurons.

10      46. A method of treating a human subject afflicted with a neurodegenerative disease which comprises introducing an amount of pharmaceutical composition of claim 41, 42, 43, or 44 effective to generating motor neurons from undifferentiated precursor cells  
15      in a human, thereby treating the human subject afflicted with a neurodegenerative disease.

20      47. The method of claim 46 wherein the generation of motor neurons from undifferentiated precursor neurons alleviates a chronic neurodegenerative disease.

25      48. The method of claim 47 wherein the chronic neurodegenerative disease is Amyotrophic lateral sclerosis (ALS).

30      49. A method of treating a human subject afflicted with an acute nervous system injury which comprises introducing an amount of pharmaceutical composition of claim 41, 42, 43, or 44 effective to generate motor neurons from undifferentiated precursor cells in a human, thereby treating a human subject afflicted with an acute nervous system injury.

35      50. The method of claim 49 wherein the acute nervous

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system injury is localized to a specific central axon which comprises surgical implantation of a pharmaceutical compound comprising a vhh-1 protein and a pharmaceutically acceptable carrier effective to generate motor neurons from undifferentiated motor neurons located proximal to the injured axon, thereby alleviating the acute nervous system injury localized to a specific central axon.

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FIGURE 1-1
FIGURE 1-2
FIGURE 1-3

## FIGURE 1-1

```

TTAAAATCAG GCTCTTTTTG TCTTTTAATT GCCGTCTCGA GACCCAACTC CGATGTGTTC 60
CGTTACCAGC GACCGGCAGC CTGCCATCGC AGCCCCTGTC TGGGTGGGGA TCGGAGACAA 120
GTCCCCTGCA GCAACAGCAG GCAAGGTTAT ATAGGAAGAG AAAGAGCCAG GCAGCGCCAG 180
AGGGAACGAA CGAGCCGAGC GAGGAAGGGA GAGCCGAGCG CAAGGAGGAG CGCACACGCA 240
CACACCCGCG CGTACCAGCT CGCGCACAGA CCGGCGCGGG GACGGCTCGC AAGTCCTCAG 300
GTTCCGCGGA CGAG ATG CTG CTG CTG CTG GCC AGA TGT TTT CTG GTG GCC 350
                Met Leu Leu Leu Leu Ala Arg Cys Phe Leu Val Ala
                1          5          10

CTT GCT TCC TCG CTG CTG GTG TGC CCC GGA CTG GCC TGT GGG CCC GGC 398
Leu Ala Ser Ser Leu Leu Val Cys Pro Gly Leu Ala Cys Gly Pro Gly
    15          20          25

AGG GGG TTT GGA AAG AGG CAG CAC CCC AAA AAG CTG ACC CCT TTA GCC 446
Arg Gly Phe Gly Lys Arg Gln His Pro Lys Lys Leu Thr Pro Leu Ala
    30          35          40

TAC AAG CAG TTT ATC CCC AAC GTA GCC GAG AAG ACC CTA GGG GCC AGC 494
Tyr Lys Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser
    45          50          55

GGC CGA TAT GAA GGG AAG ATC ACA AGA AAC TCC GAA CGA TTT AAG GAA 542
Gly Arg Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu
    65          70          75

CTC ACC CCC AAT TAC AAC CCC GAC ATC ATA TTT AAG GAT GAG GAA AAC 590
Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
    80          85          90

ACT GGA GCA GAC CGG CTG ATG ACT CAG AGG TGC AAA GAC AAG TTA AAT 638
Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn
    95          100          105

GCC TTG GCC ATC TCC GTG ATG AAC CAG TGG CCT GGA GTG AAG CTT CGA 686
Ala Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
    110          115          120

GTG ACT GAG GGC TGG GAT GAG GAC GGC CAT CAT TCA GAG GAG TCT CTA 734
Val Thr Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
    125          130          135          140

CAC TAT GAG GGT CGA GCA GTG GAC ATC ACC ACG TCT GAC AGG GAC CGC 782
His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg
    145          150          155

AGC AAG TAT GGC ATG CTG GCT CGC CTG GCT GTG GAG GCT GGA TTC GAC 830
Ser Lys Tyr Gly Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp
    160          165          170

```

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FIGURE 1-2

TGG	GTC	TAC	TAT	GAA	TCC	AAA	GCT	CGC	ATC	CAC	TGC	TCT	GTG	AAA	GCA	878
Trp	Val	Tyr	Tyr	Glu	Ser	Lys	Ala	Arg	Ile	His	Cys	Ser	Val	Lys	Ala	
		175					180					185				
GAG	AAC	TCC	GTG	GCG	GCC	AAA	TCT	GAC	GGC	TGC	TTC	CCG	GGA	TCA	GCC	926
Glu	Asn	Ser	Val	Ala	Ala	Lys	Ser	Asp	Gly	Cys	Phe	Pro	Gly	Ser	Ala	
	190					195					200					
ACA	GTG	CAC	CTG	GAG	CAG	GGT	GGC	ACC	AAG	TTA	GTG	AAG	GAT	CTA	AGT	974
Thr	Val	His	Leu	Glu	Gln	Gly	Gly	Thr	Lys	Leu	Val	Lys	Asp	Leu	Ser	
	205				210					215					220	
CCC	GGG	GAC	CGC	GTG	CTG	GCG	GCT	GAC	GAC	CAG	GGC	CGG	CTG	CTG	TAC	1022
Pro	Gly	Asp	Arg	Val	Leu	Ala	Ala	Asp	Asp	Gln	Gly	Arg	Leu	Leu	Tyr	
				225					230					235		
AGC	GAC	TTC	CTC	ACC	TTC	CTG	GAC	CGC	GAC	GAA	GGT	GCC	AAG	AAG	GTC	1070
Ser	Asp	Phe	Leu	Thr	Phe	Leu	Asp	Arg	Asp	Glu	Gly	Ala	Lys	Lys	Val	
			240					245					250			
TTC	TAC	GTG	ATC	GAG	ACG	CGG	GAG	CCG	CGG	GAG	CGT	CTG	CTG	CTC	ACT	1118
Phe	Tyr	Val	Ile	Glu	Thr	Arg	Glu	Pro	Arg	Glu	Arg	Leu	Leu	Leu	Thr	
		255					260					265				
GCC	GCG	CAC	CTG	CTC	TTC	GTG	GCG	CCG	CAC	AAC	GAC	TCC	GGG	CCC	ACT	1166
Ala	Ala	His	Leu	Leu	Phe	Val	Ala	Pro	His	Asn	Asp	Ser	Gly	Pro	Thr	
	270					275					280					
CCG	GGA	CCG	AGC	CCA	CTC	TTC	GCC	AGC	CGC	GTG	CGT	CCG	GGG	CAG	CGC	1214
Pro	Gly	Pro	Ser	Pro	Phe	Ala	Ser	Arg	Val	Arg	Pro	Gly	Gln	Arg		
	285				290				295					300		
GTG	TAC	GTG	GTG	GCT	GAA	CGC	GGC	GGG	GAC	CGC	CGG	CTG	CTG	CCC	GCC	1262
Val	Tyr	Val	Val	Ala	Glu	Arg	Gly	Gly	Asp	Arg	Arg	Leu	Leu	Pro	Ala	
				305					310					315		
GCG	GTG	CAC	AGC	GTA	ACG	CTA	CGA	GAG	GAG	GCG	GCG	GGT	GCG	TAC	GCG	1310
Ala	Val	His	Ser	Val	Thr	Leu	Arg	Glu	Glu	Ala	Ala	Gly	Ala	Tyr	Ala	
			320					325					330			
CCG	CTC	ACG	GCG	GAC	GGC	ACC	ATT	CTC	ATC	AAC	CGG	GTG	CTC	GCC	TCG	1358
Pro	Leu	Thr	Ala	Asp	Gly	Thr	Ile	Leu	Ile	Asn	Arg	Val	Leu	Ala	Ser	
		335					340					345				
TGC	TAC	GCA	GTC	ATC	GAG	GAG	CAC	AGC	TGG	GCA	CAC	CGG	GCC	TTC	GCG	1406
Cys	Tyr	Ala	Val	Ile	Glu	Glu	His	Ser	Trp	Ala	His	Arg	Ala	Phe	Ala	
	350					355					360					
CCC	TTC	CGC	CTG	GCG	CAC	GCG	CTG	CTG	GCC	GCG	CTG	GCA	CCC	GCC	CGC	1454
Pro	Phe	Arg	Leu	Ala	His	Ala	Leu	Leu	Ala	Ala	Leu	Ala	Pro	Ala	Arg	
	365				370					375					380	
ACG	GAC	GGC	GGG	GGC	GGG	GGC	AGC	ATC	CCT	GCC	CCG	CAA	TCT	GTA	GCG	1502
Thr	Asp	Gly	Gly	Gly	Gly	Gly	Ser	Ile	Pro	Ala	Pro	Gln	Ser	Val	Ala	
				385					390					395		
GAA	GCG	AGG	GGC	GCA	GGG	CCG	CCT	GCG	GCG	ATC	CAC	TGG	TAC	TCG	CAG	1550
Glu	Ala	Arg	Gly	Ala	Gly	Pro	Pro	Ala	Gly	Ile	His	Trp	Tyr	Ser	Gln	
		400						405					410			

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### FIGURE 1-3

CTG	CTG	TAC	CAC	ATT	GGC	ACC	TGG	CTG	TTG	GAC	AGC	GAG	ACC	CTG	CAT	1598				
Leu	Leu	Tyr	His	Ile	Gly	Thr	Trp	Leu	Leu	Asp	Ser	Glu	Thr	Leu	His	.				
		415					420					425								
CCC	TTG	GGA	ATG	GCA	GTC	AAG	TCC	AGC	TGAAGTCCGA	CGGGACCGGG						1645				
Pro	Leu	Gly	Met	Ala	Val	Lys	Ser	Ser												
		430				435														
CAGGGGGCGT															GGGGGCGGGC	GGGGCGGGAA	GCGACTGCCA	GATAAGCAAC	CGGGAAAGCG	1705
CACGGAAGGA																				1715

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FIGURE 2A-1

FIGURE 2A-1
FIGURE 2A-2

1	hh	MDNHSSVPWA	SAASVTCLSL	DAKCHSSSSS	SSSKSAASSI	SAIPQEETQT
51	zf	...	.MRLLTRVLL	VSLI.TLSLVV	SGLACGPGRG	YGRRRHPKKL
	R	...	MLLLLARCFL	VALASSLLVC	PGLACGPGRG	FGKRQHPKKL
	hh	MRHIAHTQRC	LSRLTSLVAL	LLI'VLP MVES	PAHSCGPGRG	LG.RHRARNL
101	zf	TPLAYKQFIP	NVAEKTLCAS	GRYIGKITRN	SERFKELTPN	YNPDIIFKDE
	R	TPLAYKQFIP	NVAEKTLCAS	GRYIGKITRN	SERFKELTPN	YNPDIIFKDE
	hh	YPLVLKQTIP	NLSEYTNAS	GPLEGVIRRD	SPKFKDLVPN	YNRDI LFRDE
151	zf	ENTGADRIMT	QRCKDKLNSL	AISVMNHWP	VKLRVTEGWD	EDGHHFEESL
	R	ENTGADRIMT	QRCKDKLNSL	AISVMNQWP	VKLRVTEGWD	EDGHHFEESL
	hh	EGTGADRIMS	KRCCKEKLNVL	AYSVMNEWPG	IRLLVTESWD	EDYHHGQESL
201	zf	HYEGRVDIT	TSDRDKSKYG	TLRLAVEAG	FDWVYYESKA	HIHCSVKAEN
	R	HYEGRVDIT	TSDRDKSKYG	MLARLAVEAG	FDWVYYESKA	RIHCSVKAEN
	hh	HYEGRVTIA	TSDRDQSKYG	MLARLAVEAG	FDWVSYSVSR	HIYCSVKSDS

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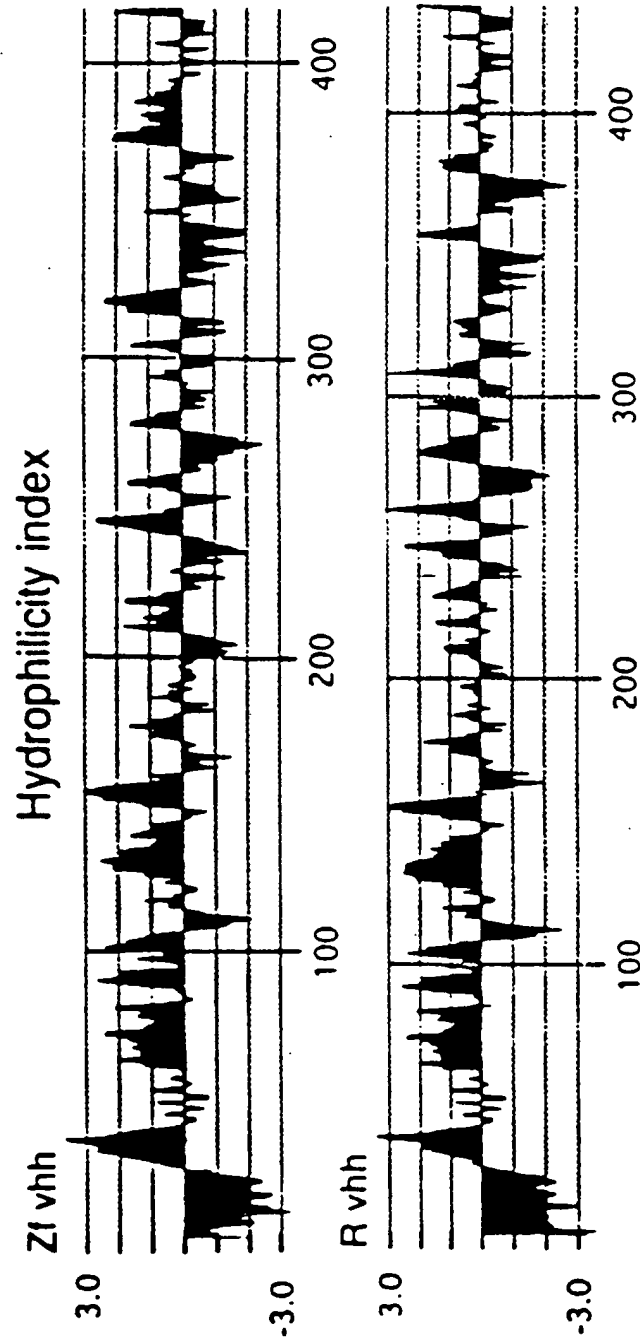
## FIGURE 2A-2

```

251
zf vhh SVAAKSGGCF PCSALVSLQD GGQKAVKDLN PGDKVLAADS AGNLVFSDFI
R vhh SVAAKSDGCF PGSATVHLEQ GGTIKLVKDLN PGDRVLAADD QGRLIYSDFL
hh SISSHVHGCF TPESTALLES GVRKPLGELS IGDRVLSMTA NGQAVYSEVI
301
zf vhh MFTDRDSTTR RVFYVIETQE PVEKITLTAA HLLFVL.DNS TEDLHTMTAA
R vhh TFLDRDEGAK KVFYVIETRE PRERLLITAA HLLFVAFPHND SGPTPGPSPL
hh LFMDRNLEQM QNFVQLHT.D GGAVLTVTPA HLVSVWQPES ....QKLT.FV
351
zf vhh YASSVRAGOK VMVVD.DSGQ LKSIVVQRIY T....EEQRG SFAPVTAHGT
R vhh FASRVRFGR VYVVA.ERGG DRRLLPAAVH SVTLREEAAG AYAPLTADGT
hh FADRIEKNQ VLVRDVETGE LRPQRVVKVG SV....RSKG VVAPLTREFGT
401
zf vhh IVVDRILASC YAVIEDQGIA HLAFAFARLY YVVSFLFPQ NSSRSRNLTL
R vhh ILINRVLASC YAVIEEHSWA HRAFAFRLA HALLAALAPA RTDGGGGSI
hh IVVNSVAASC YAVINSQSLA HWGLAPMRLI STLEAWLPK EQLHSSPKVV
451
zf vhh ..... QQEGVHW YSRLLYQMGT WLDSNMLHP LGMSVNSS*
R vhh PAQSVAEAR GAGPPAGIHW YSOLLXHGHT WLDSNMLHP LGMAVKSS*
hh SSAQ..... QQNGIHW YANALYKVKD YVLPQSWRHD *
```



FIGURE 2B



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FIGURE 3A

FIGURE 3B

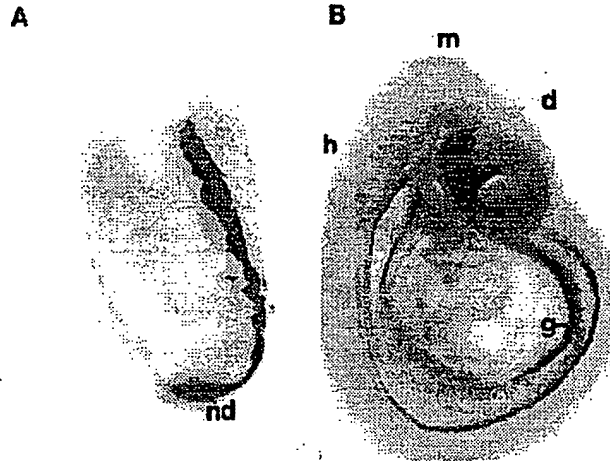


FIGURE 3C

FIGURE 3D

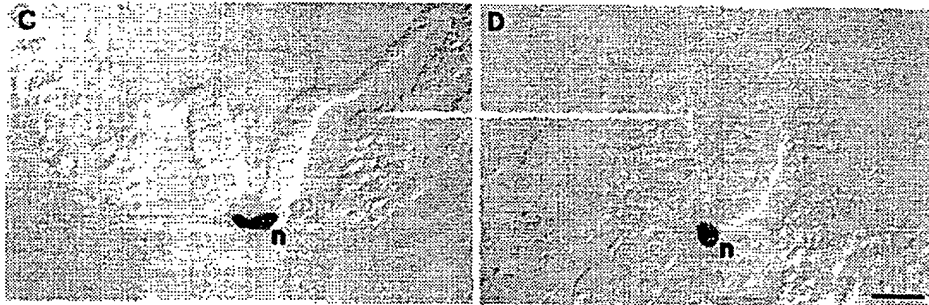


FIGURE 3E

E



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FIGURE 4C

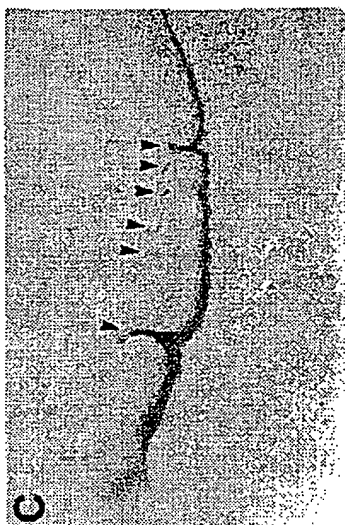


FIGURE 4B

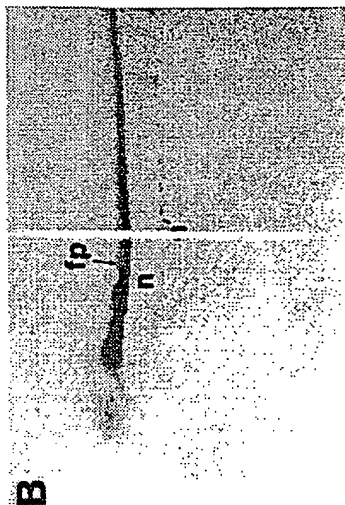


FIGURE 4A

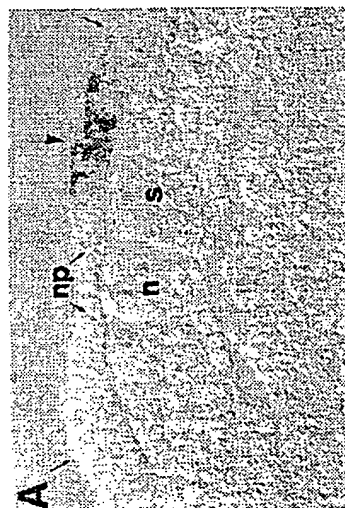


FIGURE 4F

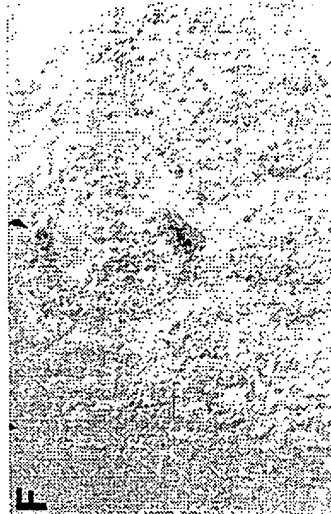


FIGURE 4E

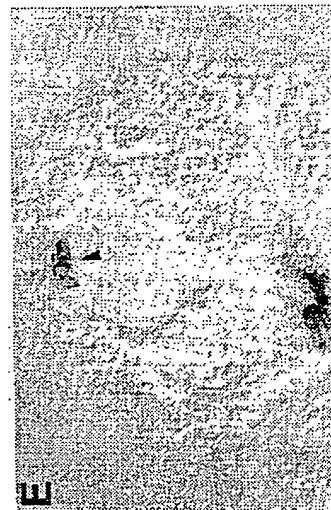
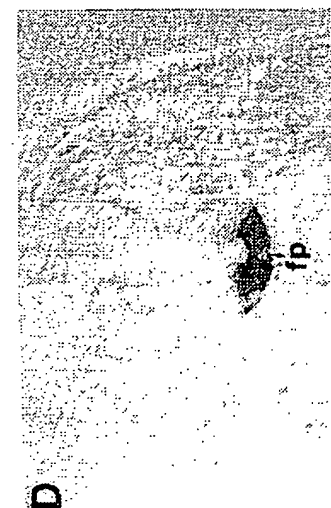


FIGURE 4D



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FIGURE 4I

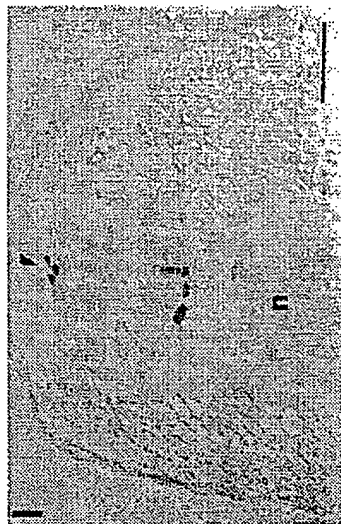


FIGURE 4H

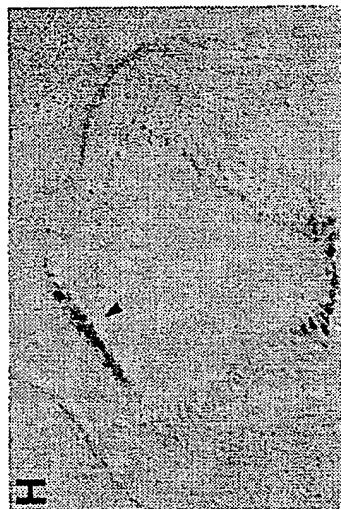
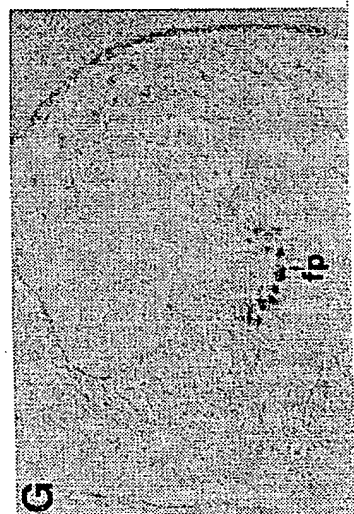


FIGURE 4G



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FIGURE 5A

FIGURE 5B



FIGURE 5C

FIGURE 5D

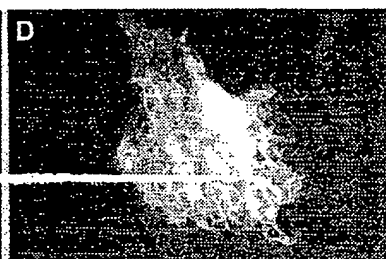
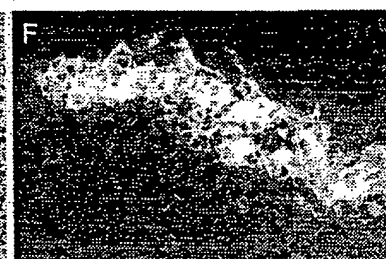
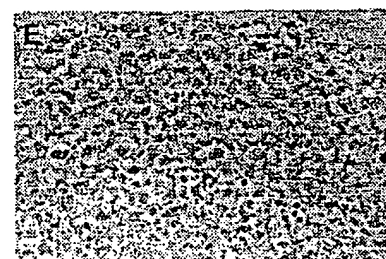


FIGURE 5E

FIGURE 5F

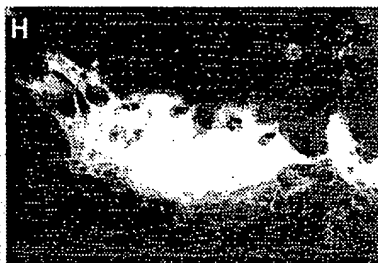


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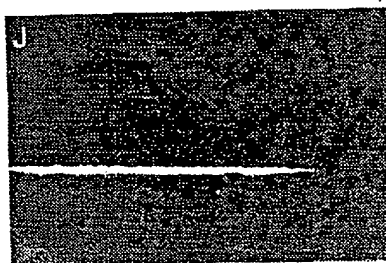
**FIGURE 5G**



**FIGURE 5H**



**FIGURE 5J**



**FIGURE 5K**



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FIGURE 6A

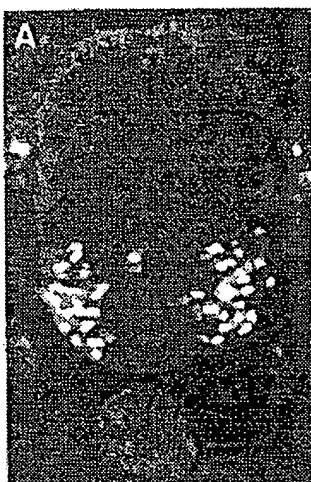


FIGURE 6B



FIGURE 6C

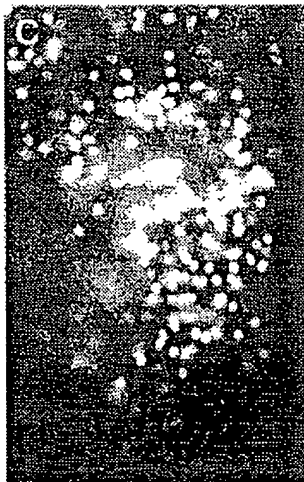


FIGURE 6D

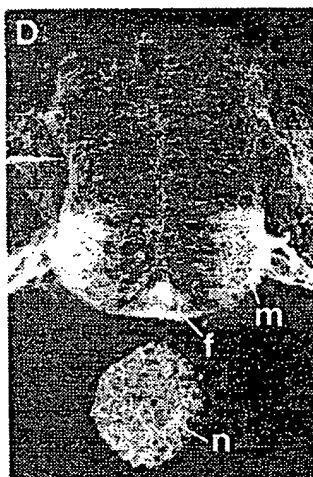


FIGURE 6E



FIGURE 6F

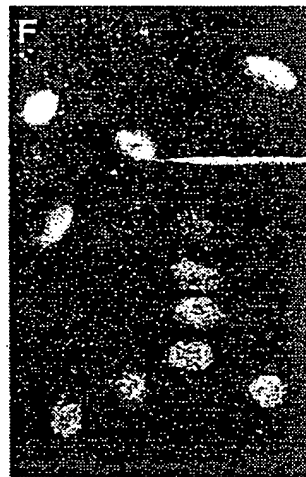
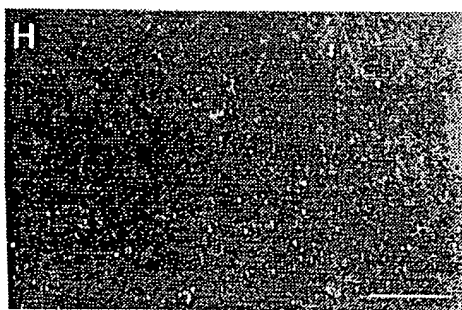


FIGURE 6G



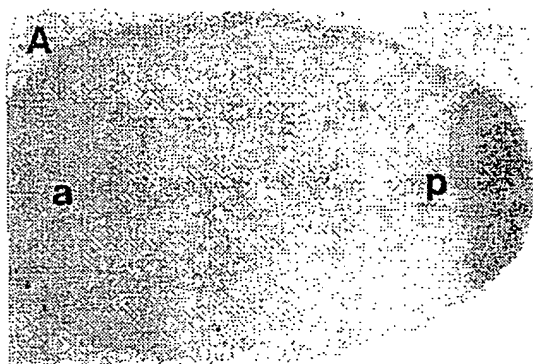
FIGURE 6H



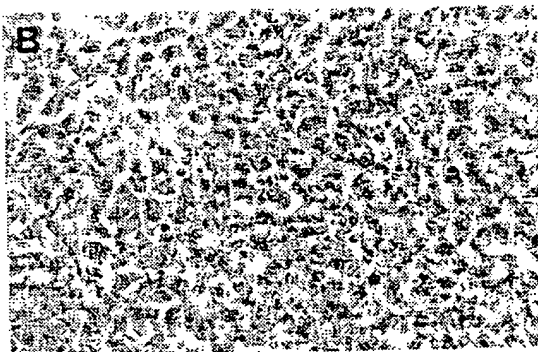
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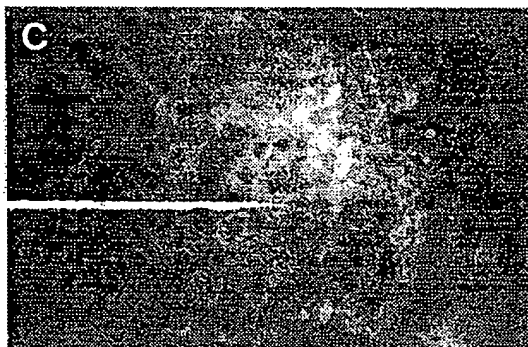
**FIGURE 7A**



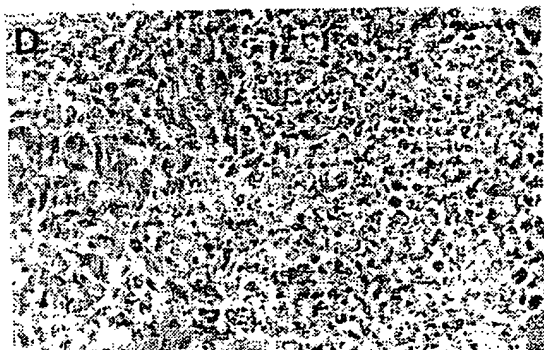
**FIGURE 7B**



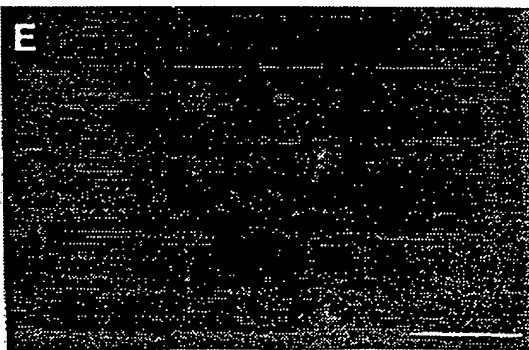
**FIGURE 7C**



**FIGURE 7D**



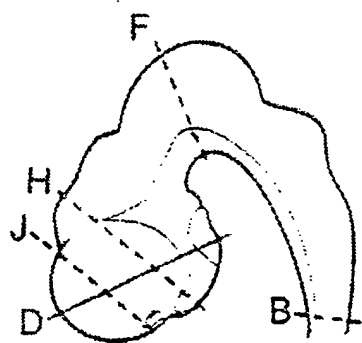
**FIGURE 7E**





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FIGURE 8A



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FIGURE 8C

FIGURE 8D

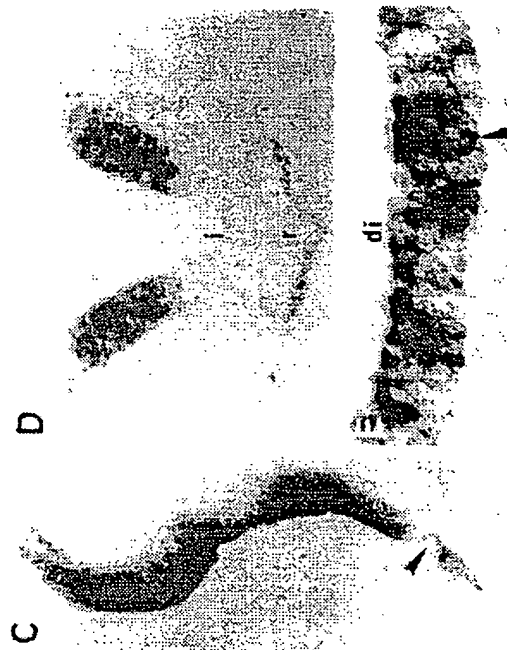


FIGURE 8E

FIGURE 8B



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FIGURE 8G



FIGURE 8I

FIGURE 8F

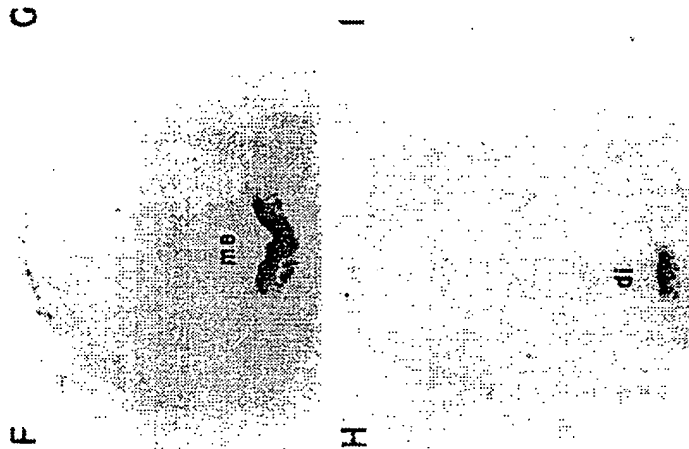


FIGURE 8H

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FIGURE 8K



K

FIGURE 8J

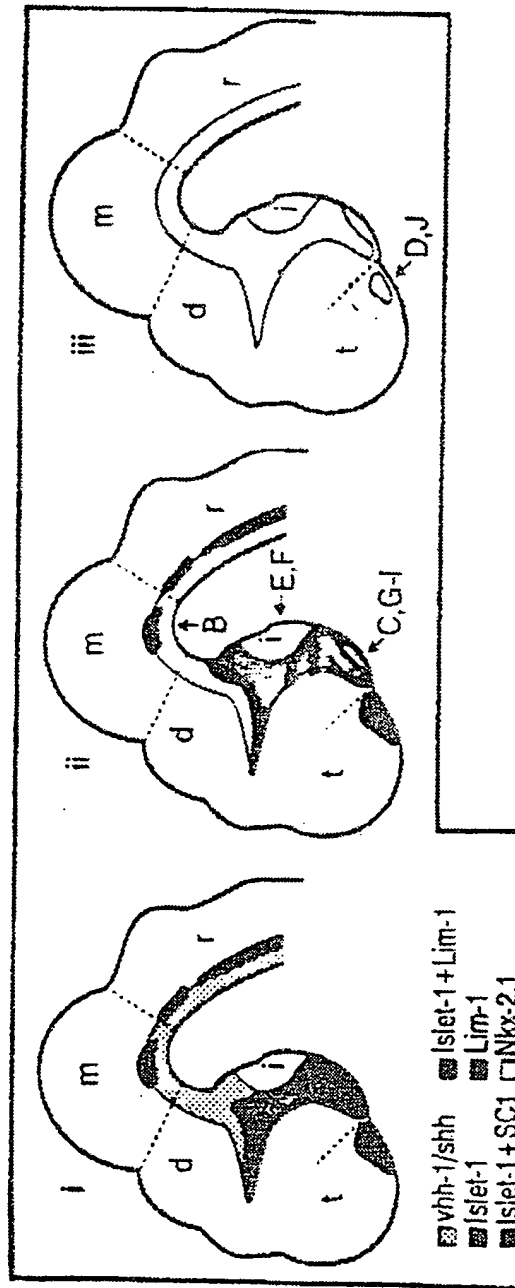


J

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FIGURE 9A



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FIGURE 9B

FIGURE 9C

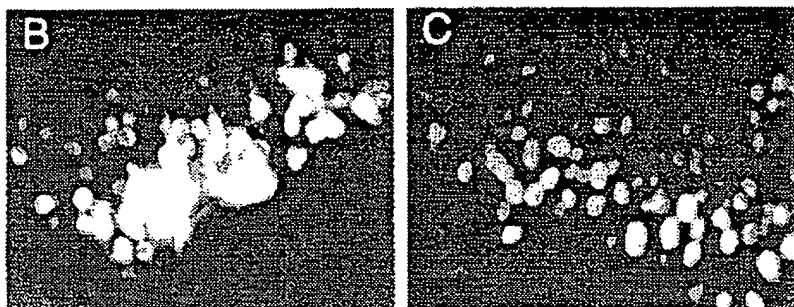


FIGURE 9D



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FIGURE 9E

FIGURE 9F

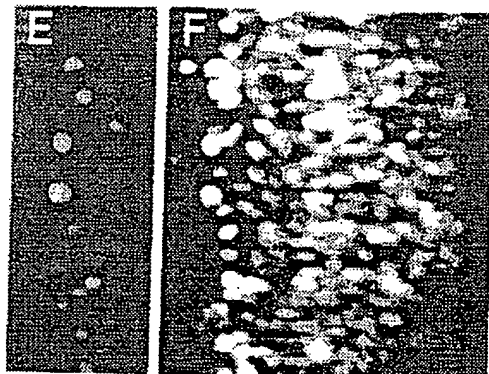


FIGURE 9H

FIGURE 9G

FIGURE 9I

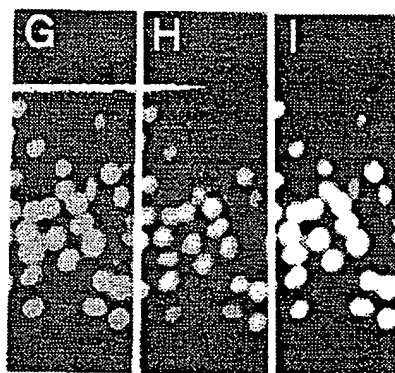
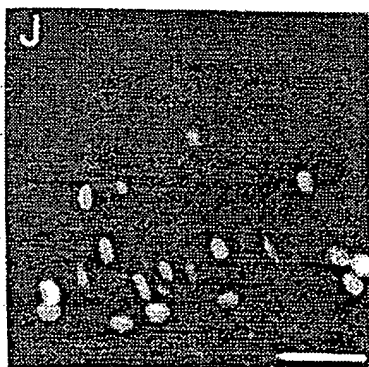


FIGURE 9J



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FIGURE 10B

FIGURE 10F

FIGURE 10A

FIGURE 10D

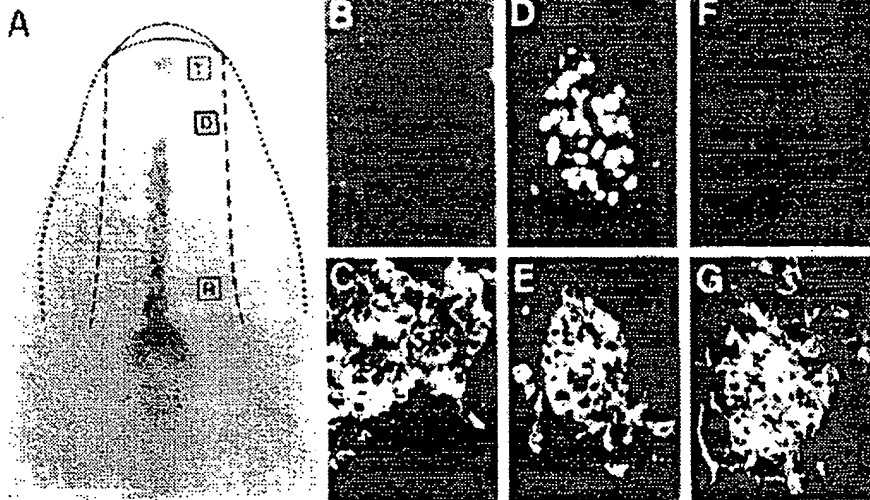


FIGURE 10C

FIGURE 10G

FIGURE 10E

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FIGURE 10J

FIGURE 10H

FIGURE 10L

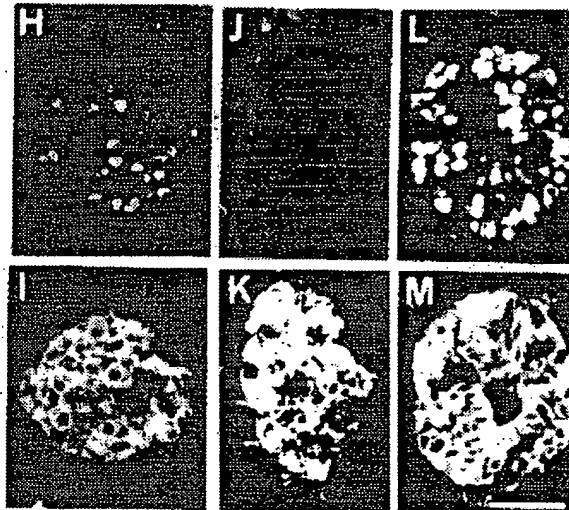


FIGURE 10I

FIGURE 10M

FIGURE 10K

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FIGURE 11G

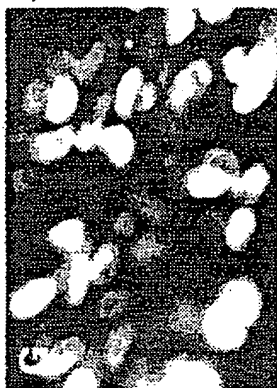


FIGURE 11E

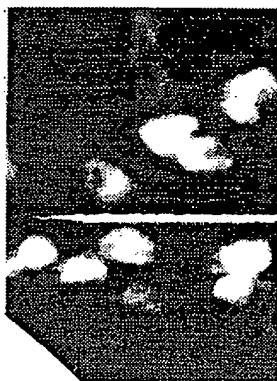


FIGURE 11C



FIGURE 11A

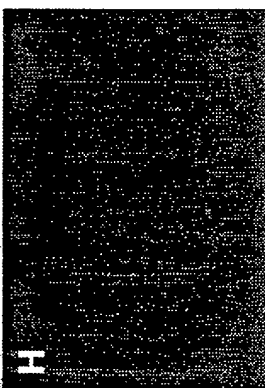
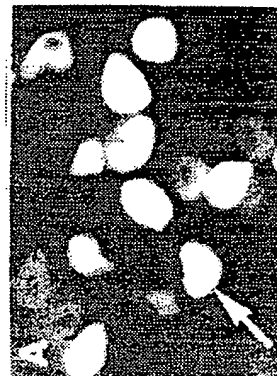


FIGURE 11H



FIGURE 11F



FIGURE 11D



FIGURE 11B

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FIGURE 12A FIGURE 12B FIGURE 12C

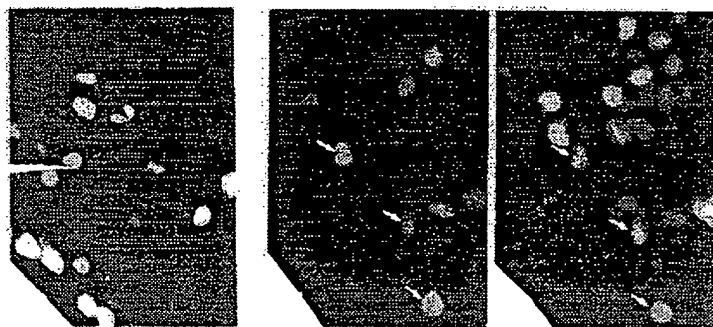
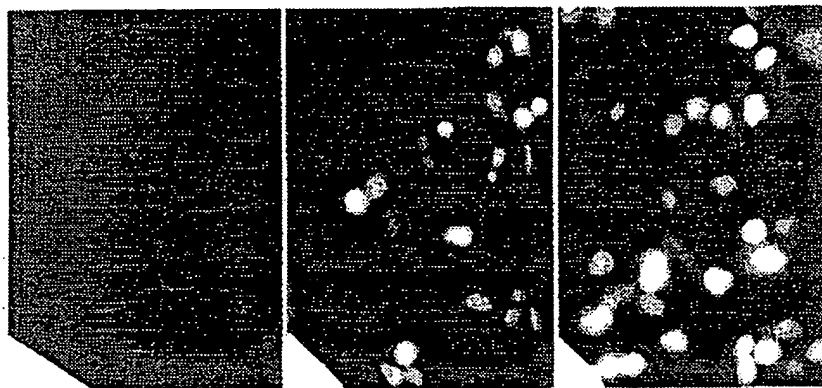
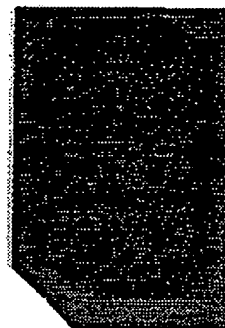
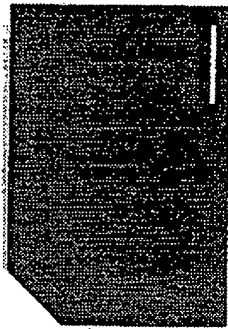


FIGURE 12D FIGURE 12E FIGURE 12F

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FIGURE 12G FIGURE 12H FIGURE 12I



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FIGURE 13A

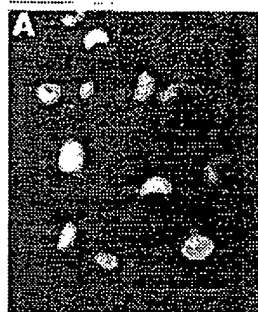


FIGURE 13B



FIGURE 13C

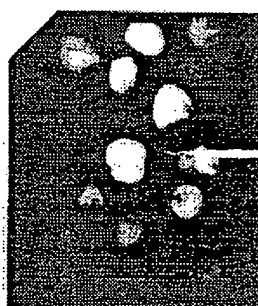
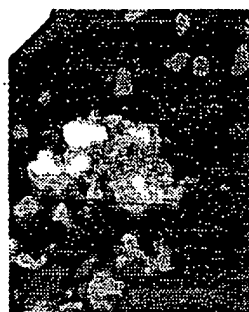


FIGURE 13D

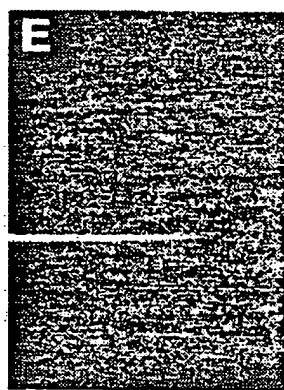


FIGURE 13E

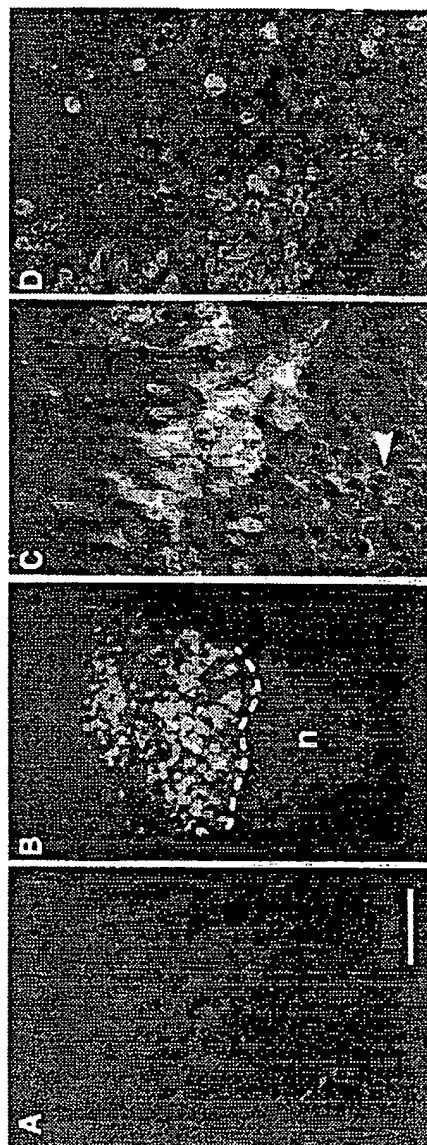


FIGURE 13F

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FIGURE 14A FIGURE 14B FIGURE 14C FIGURE 14D



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FIGURE 14E

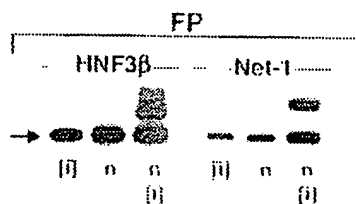
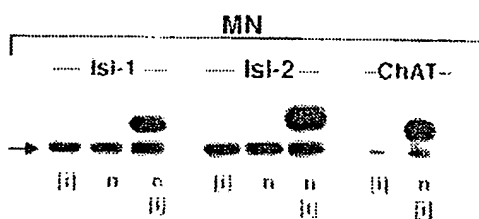
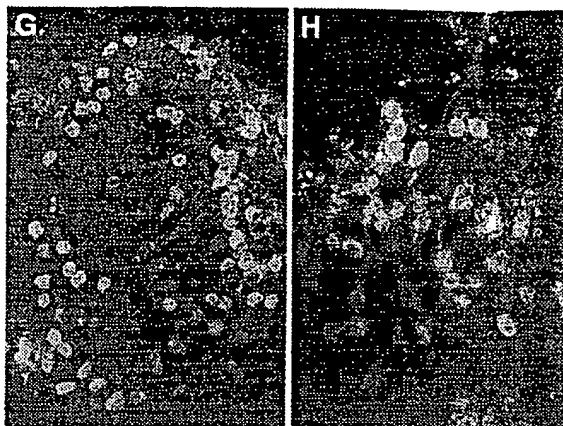


FIGURE 14F



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FIGURE 14G FIGURE 14H



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FIGURE 15A

FIGURE 15B

FIGURE 15C

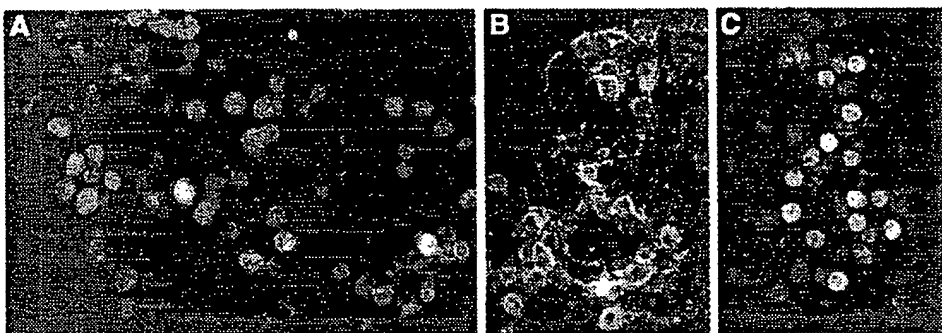
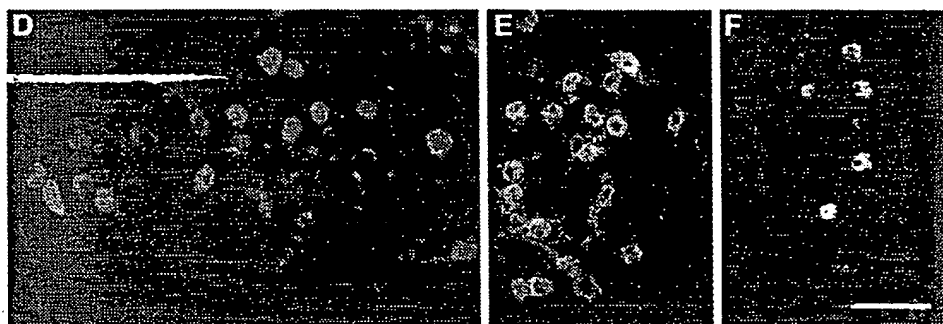


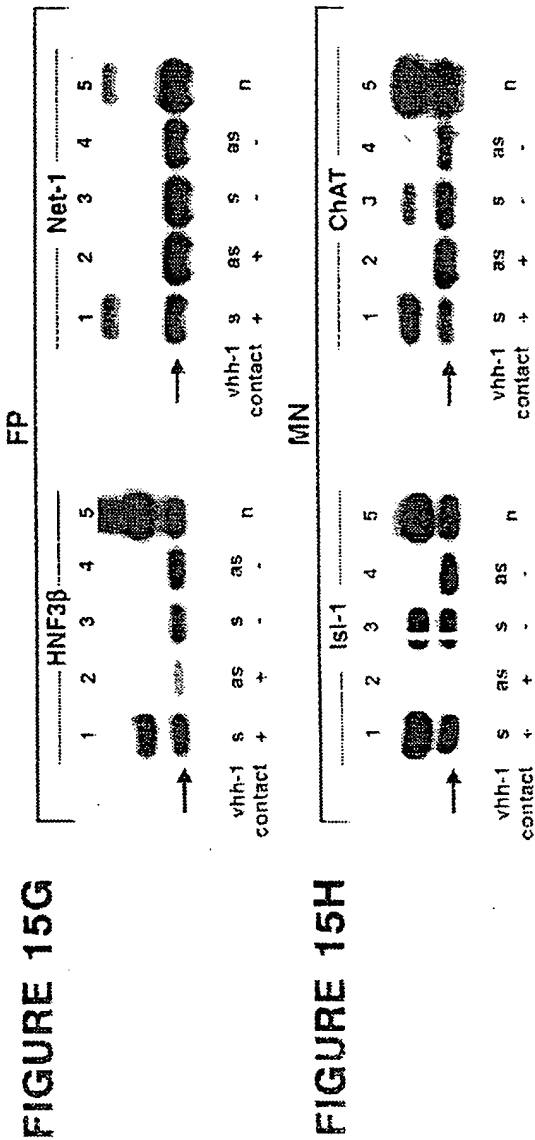
FIGURE 15D

FIGURE 15E

FIGURE 15F



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FIGURE 16B

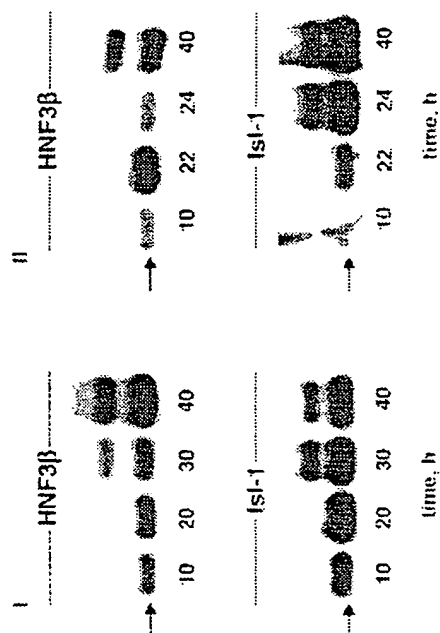
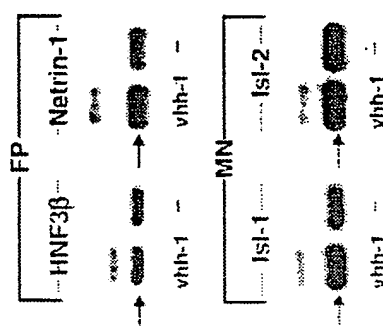


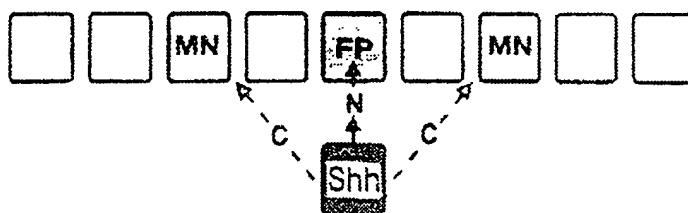
FIGURE 16A



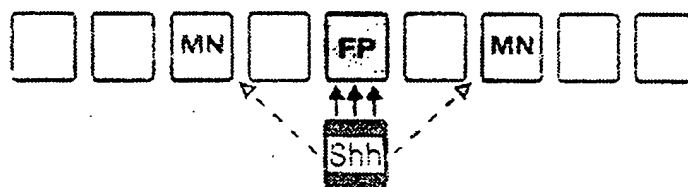
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**FIGURE 17A** floor plate and motor neuron induction by different proteolytic fragments of shh/vhh-1



**FIGURE 17B** floor plate and motor neuron induction by different concentrations of shh/vhh-1



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FIGURE 18A

FIGURE 18B

FIGURE 18C

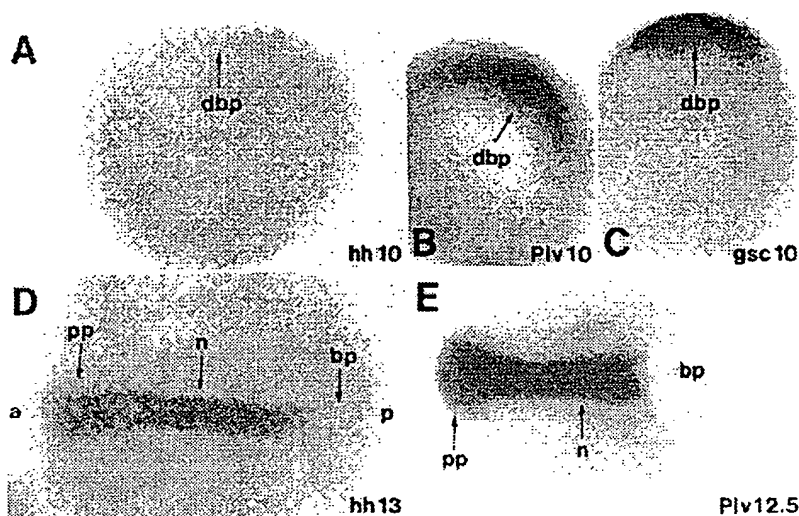


FIGURE 18D

FIGURE 18E

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FIGURE 18F

FIGURE 18G

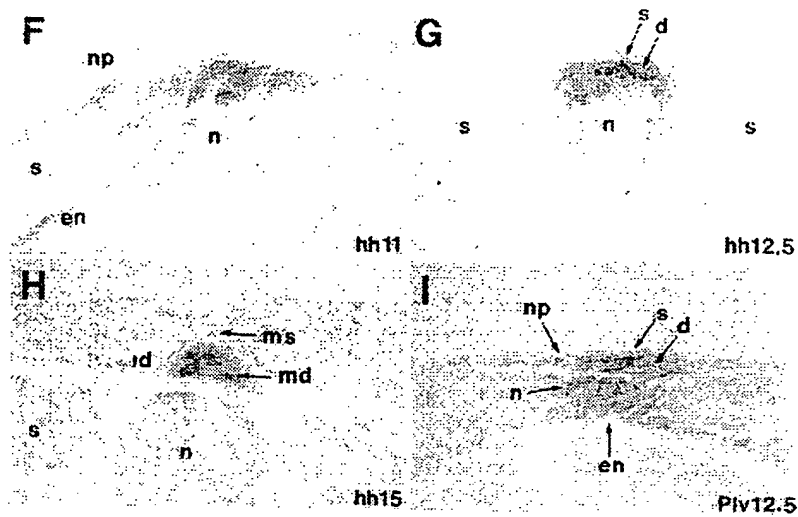


FIGURE 18H

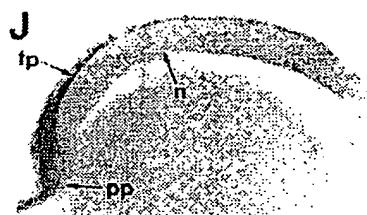
FIGURE 18I

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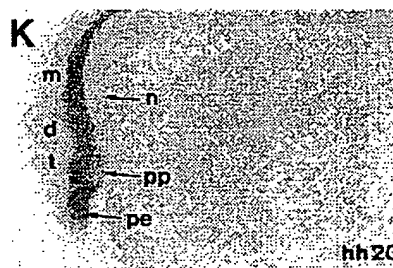
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FIGURE 18J

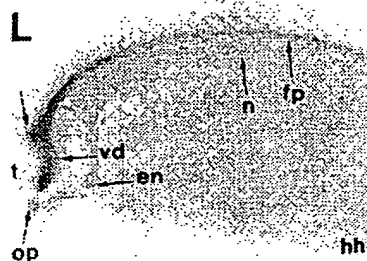
FIGURE 18K



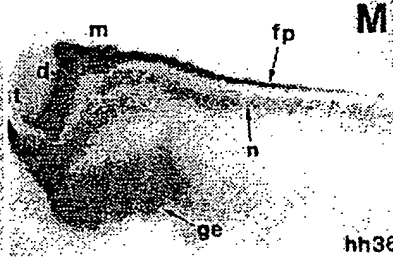
hh15



hh20



hh26



hh36

FIGURE 18L

FIGURE 18M

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FIGURE 18N

FIGURE 18P

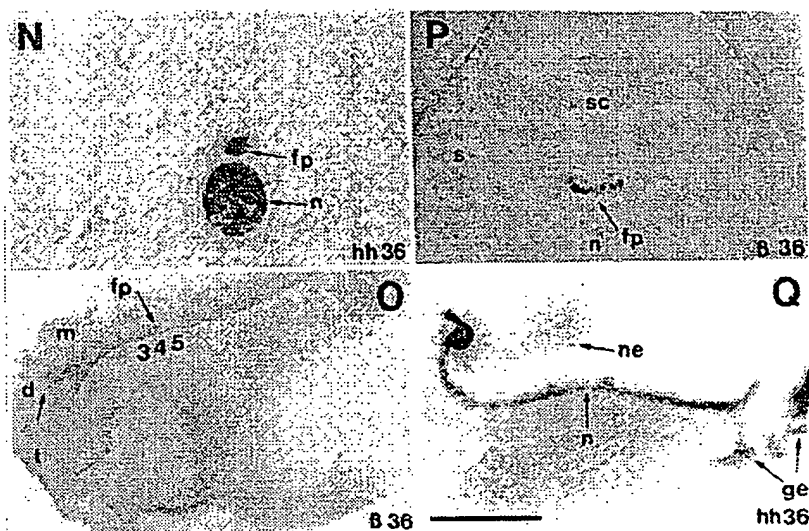


FIGURE 18O

FIGURE 18Q



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FIGURE 19A

FIGURE 19B

FIGURE 19C

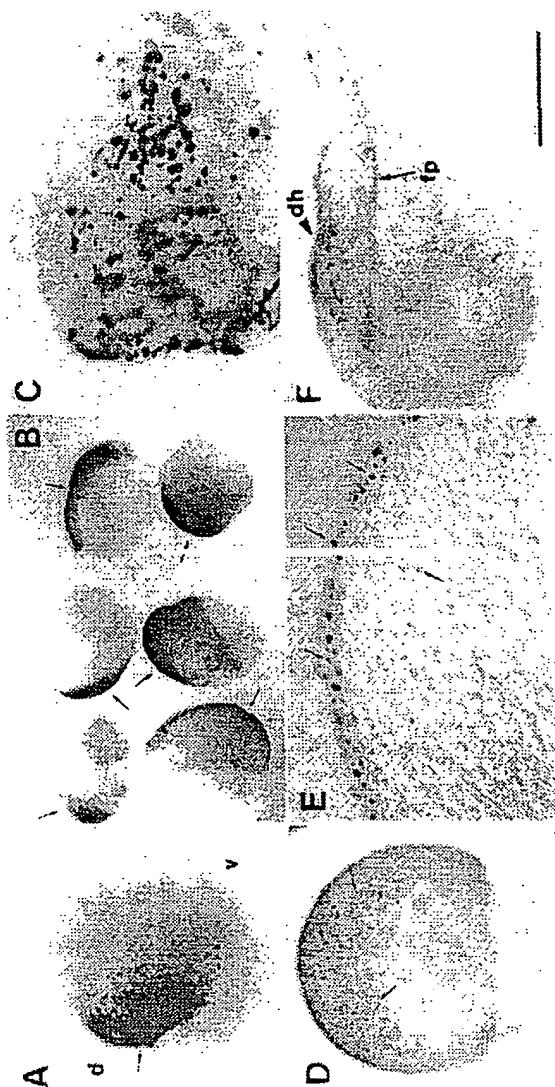


FIGURE 19F

FIGURE 19E

FIGURE 19D

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FIGURE 20A      FIGURE 20B      FIGURE 20C

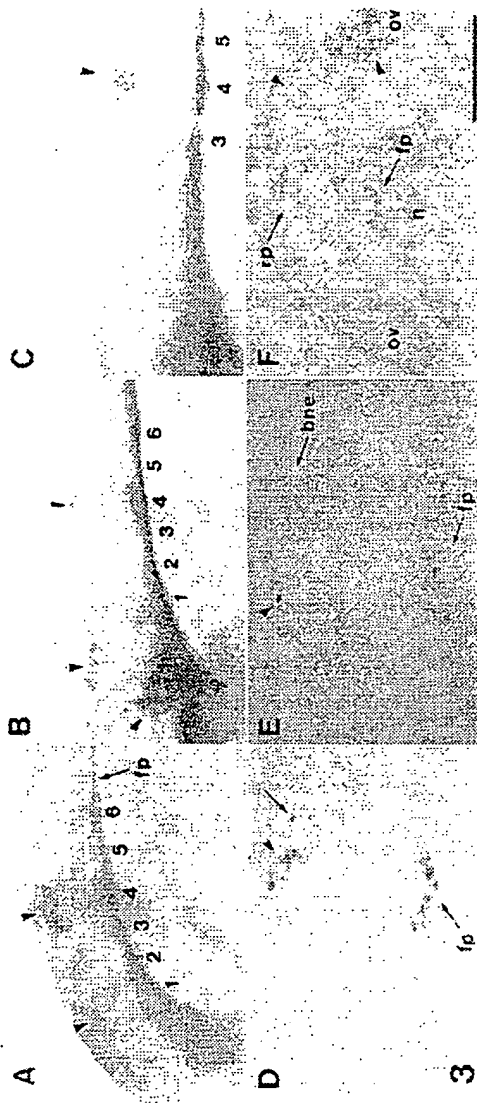


FIGURE 20D      FIGURE 20E      FIGURE 20F

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FIGURE 21A

FIGURE 21B

FIGURE 21C

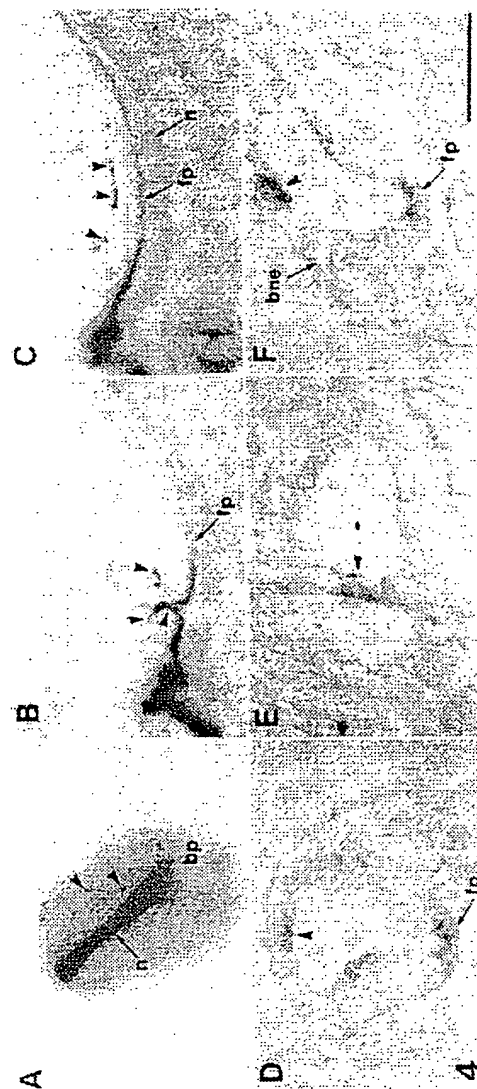


FIGURE 21F

FIGURE 21E

FIGURE 21D

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FIGURE 22A      FIGURE 22C      FIGURE 22E

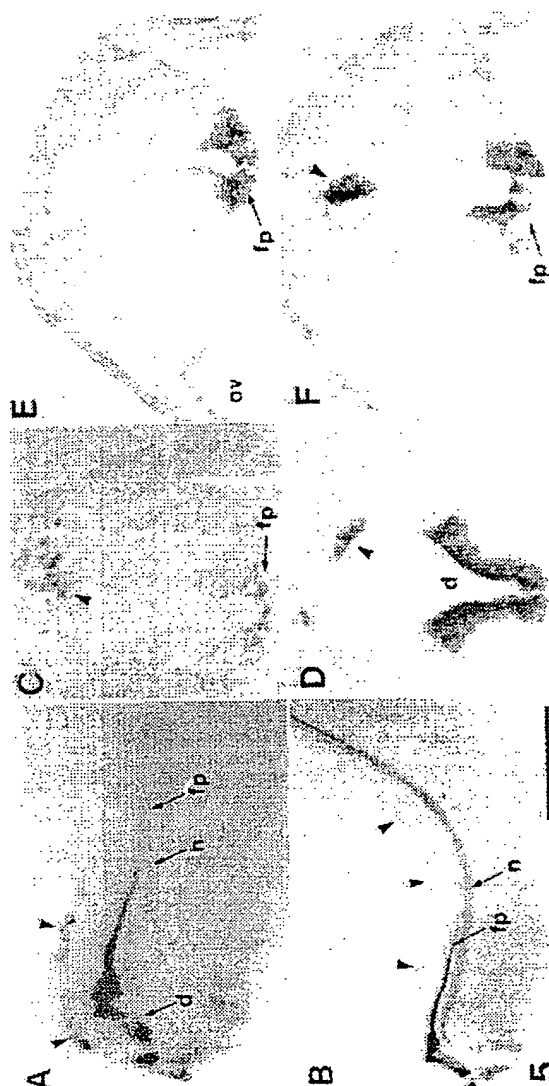


FIGURE 22B      FIGURE 22D      FIGURE 22F

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FIGURE 23A normal expression

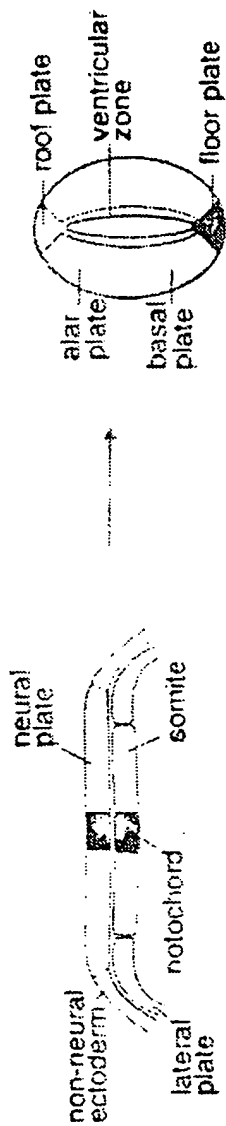


FIGURE 23B expression in injected embryos

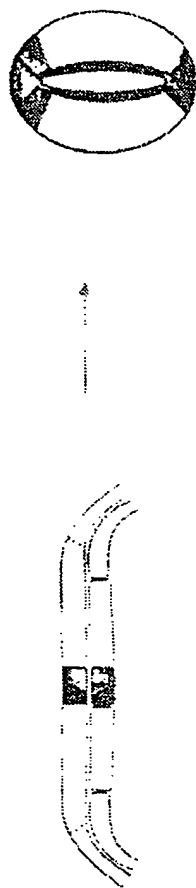
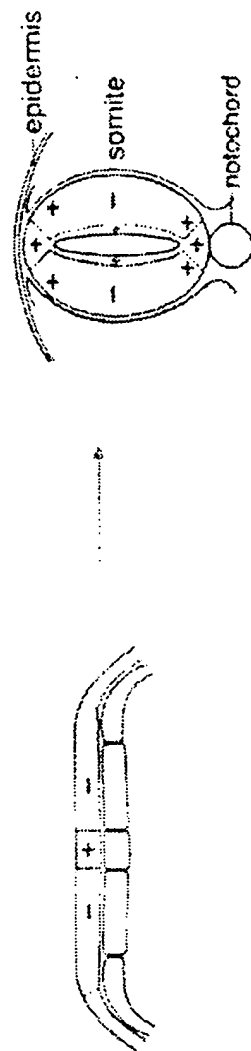


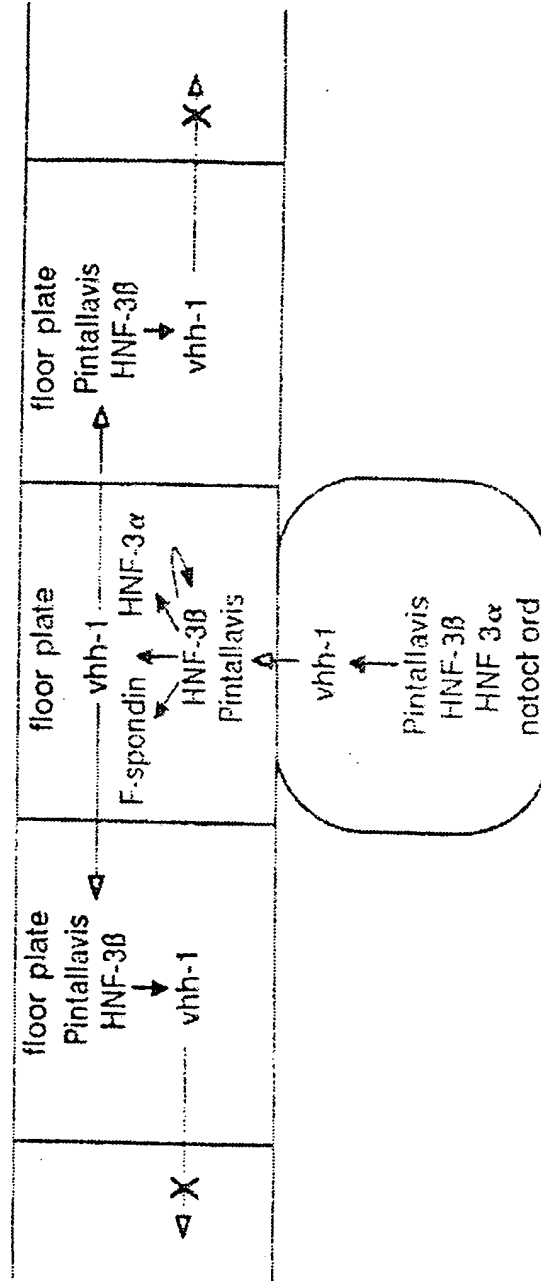
FIGURE 23C competence of neural tissue



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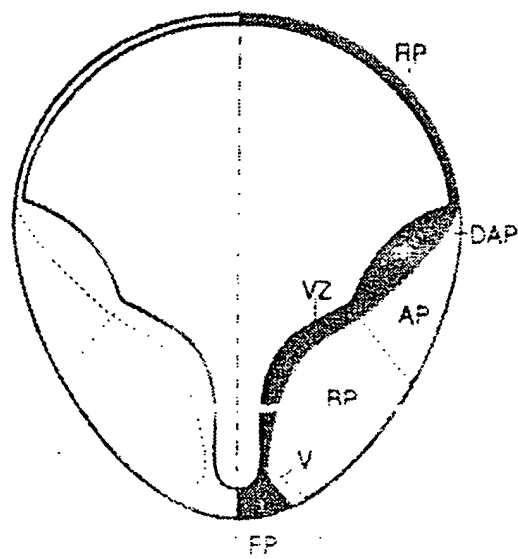
FIGURE 23D

molecular interactions



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FIGURE 24



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/02315

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5, 24.31; 435/6, 69.1, 69.4, 172.3, 240.2, 252.3, 320.1; 530/324, 350, 387.1, 399; 514/2, 12; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	CELL, Volume 75, issued 31 December 1993, R.D. Riddle et al, "Sonic hedgehog Mediates the Polarizing Activity of the ZPA", pages 1401-1416, see entire document.	1-3, 5, 8-11, 17-18 ----- 4, 6-7, 10-16, 18-24, 34 ----- 25-33, 35-50

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A document defining the general state of the art which is not considered to be of particular relevance	*X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E earlier document published on or after the international filing date	*Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*& document member of the same patent family
*O document referring to an oral disclosure, use, exhibition or other means	
*P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

18 APRIL 1995

Date of mailing of the international search report

01 JUN 1995

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/02315

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	CELL, Volume 75, issued 31 December 1993, Y. Echelard et al, "Sonic Hedgehog, a Member of a Family of Putative Signaling Molecules, Is Implicated in the Regulation of CNS Polarity", pages 1417-1430, see entire document.	1-3, 5, 8-11, 17-18 ----- 4, 6-7, 10-16, 18-24, 34 ----- 25-33, 35-50

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/02315

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/02315

## A. CLASSIFICATION OF SUBJECT MATTER: IPC (6):

C12N 15/00, 15/12; A61K 38/18; C07K 14/435, 14/46, 14/475, 16/00, 16/18, 16/22

## A. CLASSIFICATION OF SUBJECT MATTER: US CL :

536/23.5, 24.31; 435/6, 69.1, 69.4, 172.3, 240.2, 252.3, 320.1; 530/324, 350, 387.1, 399; 514/2, 12; 800/2

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS and DIALOG (files 5, 155, 351,357,358) search terms: vertebrate, hedgehog, sonic, transgenic, hybrid?, floor plate, differentiat?, motor neuron, ventral, ALS, amyotrophic lateral sclerosis

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

I. Claims 1-24 and 41-44, drawn to polynucleotide sequences, vectors, host cells, methods of production, and polypeptides, classified in at least Class 536, subclass 23.5, for example.

II. Claims 25-30, drawn to an antibody, classified in at least Class 530, subclass 387.1, for example.

III. Claim 31-34, drawn to transgenic animals and methods of determining physiologic effects, classified in at least Class 800, subclass 2, for example.

IV. Claims 35-36, drawn to methods of inducing differentiation of floor plate cells, classified in at least Class 435, subclass 240.2.

V. Claims 37-40 and 45-50, drawn to methods of treating motor neurons, classified in at least Class 514, subclass 2.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Groups I-III are drawn to structurally different products which do not share the same or a corresponding special technical feature. Groups IV-V are drawn to methods having different goals, method steps, and starting materials which do not share the same or a corresponding special technical feature. Note that PCT Rule 13 does not provide for multiple products or methods within a single application.